Universidade de Aveiro Departamento de Química
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Joana Sá Ramalho Simões

Extração e estrutura dos polissacarídeos do resíduo do café com atividade imunoestimuladora.

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Química, realizada sob a orientação científica do Doutor Manuel António Coimbra Rodrigues da Silva, Professor Associado com Agregação do Departamento de Química da Universidade de Aveiro e da Doutora Maria do Rosário Gonçalves dos Reis Marques Domingues, Professora Auxiliar do Departamento de Química da Universidade de Aveiro.

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Dedico ao Johnny e aos meus pais.

o júri

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palavras-chave

galactomanana, arabinogalactana, café, *Aloe vera*, goma de alfarroba, espetrometria de massa, torra, caracterização estrutural, atividade imunoestimuladora

resumo

As galactomananas das infusões de café apresentam atividade imunoestimuladora *in vitro*, sendo esta atividade semelhante à das mananas acetiladas extraídas de *Aloe vera*. As galactomananas presentes no resíduo de café também possuem atividade imunoestimuladora *in vitro* quando são parcialmente acetiladas. Como as galactomananas são o componente maioritário do resíduo de café e como o café é um produto de largo consumo a nível mundial, o reaproveitamento deste resíduo como fonte de galactomananas com atividade imunoestimuladora deve ser considerado. Esta dissertação procura dar resposta a duas questões: 1. Quais são as estruturas das galactomananas responsáveis pela atividade imunoestimuladora destes polissacarídeos; e 2. Como é que as galactomananas podem ser extraídas quantitativamente do resíduo de café de modo a serem solúveis em água à temperatura ambiente e, assim, poderem ser utilizadas como ingredientes alimentares com atividade imunoestimuladora.

A questão 1 foi respondida pela caracterização estrutural de quatro galactomananas, de três origens: a) as galactomananas das infusões de café e do resíduo que apresentaram atividade imunoestimuladora; b) a galactomanana da goma de alfarroba (LBG), que não apresentou atividade imunoestimuladora; e c) a manana acetilada de *Aloe vera*, que apresentou atividade imunoestimuladora. Estes polissacarídeos foram submetidos à análise de açúcares e de ligações glicosídicas e a hidrólise por *endo*-β-D-(1→4)-mananase. Os fragmentos de oligossacarídeos mais pequenos foram ainda analisados por espetrometria de massa por ionização de electrospray e espetrometria de massa tandem.

As galactomananas das infusões de café, do resíduo de café e do *Aloe vera* apresentaram grau de ramificação e peso molecular semelhantes, enquanto as galactomananas da LBG apresentaram grau de ramificação e de polimerização maiores. Todas as galactomananas apresentaram resíduos de arabinose como ramificação. O grau de acetilação das galactomananas da LBG foi vestigial enquanto as galactomananas do *Aloe vera* apresentaram um grau de acetilação de 2,08; para as galactomananas do resíduo de café o grau de acetilação foi de 0,98 e para as infusões foi de 0,08. A localização dos grupos acetilo foi irregular em todos os polímeros. Os resultados obtidos permitem inferir que baixos níveis de ramificação, cadeias pequenas e alguma acetilação parecem promover a atividade imunoestimuladora atribuída às galactomananas.

Para responder à questão 2, foi testada uma metodologia que envolveu a torra do resíduo de café a 160 ºC e a 220 ºC e a sua extração com água quente e com soluções de 4 M NaOH à temperatura de 20, 60 e 120 ºC. A torra do resíduo a 160 ºC e a extração sequencial permitiu extrair 56% das galactomananas presentes no resíduo de café e, simultaneamente, 54% das arabinogalactanas. As galactomananas mantiveram a sua estrutura caraterística de polissacarídeo acetilado composto por uma cadeira principal de resíduos de manose em ligação β-(1$\rightarrow$4) e resíduos de Gal e Ara nas cadeias laterais. A 220 ºC, as galactomananas foram parcialmente degradadas e o rendimento de extração foi muito menor do que a 160 ºC. No entanto, mesmo a esta temperatura as galactomananas apresentaram resíduos acetilados e a presença de pentoses nas cadeias laterais, o que permite inferir a elevada resistência destes polissacarídeos à temperatura e aos reagentes alcalinos.

De forma a melhor compreender a estabilidade térmica das galactomananas do resíduo de café e a influência que a presença de arabinogalactanas pode ter na sua estabilidade, foi feita uma análise termogravimétrica aos polissacarídeos extraídos do resíduo de café assim como a polissacarídeos relacionados estruturalmente com estes, como a celulose, a galactomanana de LBG e a goma arábica, uma arabinogalactana. As galactomananas são termicamente estáveis durante 3 h a 200 ºC, enquanto as arabinogalactanas são estáveis a 180 ºC. De acordo com os perfis dos termogramas obtidos, e pelo cálculo das energias de ativação da degradação térmica, o resíduo de café apresenta uma estabilidade térmica menor do que a galactomanana, possivelmente devido à presença de arabinogalactanas. Apesar de não se ter verificado alterações no termograma da galactomanana do café submetida a um tratamento térmico de 200 ºC durante 3 h, verificam-se alterações estruturais que envolvem a formação de novas ligações glicosídicas, nomeadamente, a formação de resíduos de manose ligados em O-2 e em O-6, reações de transglicosilação, despolimerização, formação de resíduos de anidro-hexoses no terminal redutor e isomerização manose-glucose. Estas alterações promovem a solubilização das galactomananas.

Os resultados obtidos permitem propor que o resíduo de café possa ser submetido a uma torra seguida de extração com reagentes alcalinos a quente para obtenção das galactomananas com rendimentos elevados. Estes polissacarídeos podem tornar-se solúveis em água após tratamento térmico a 200 ºC, permitindo assim a sua utilização em formulações alimentares, nomeadamente, por preparação de compostos acetilados com baixos níveis de ramificação e cadeias pequenas de modo a promover a sua atividade imunoestimuladora.

keywords

galactomannan, arabinogalactan, coffee, *Aloe vera*, Locust bean gum, mass spectrometry, roasting, structural characterization, immunostimulatory activity

abstract

The coffee infusion galactomannans present *in vitro* immunostimulatory activity. These polysaccharides present a similar activity to the acetylated galactomannans of *Aloe vera*. The galactomannans extracted from coffee by-product, when partially acetylated, also present *in vitro* immunostimulatory activity. As the galactomannans are the main components of coffee residue, and because the coffee is a product largely consumed all over the world, the reutilization of the residue as a source of galactomannans with immunostimulatory activity required to be considered. The present PhD thesis tries to answer two questions: 1. What the galactomannans structures responsible for the immunostimulatory activities of these polysaccharides are; and 2. How can the galactomannans be quantitatively extracted from coffee residue to be rendered soluble in water at room temperature and, thus, be used as food ingredients with immunostimulatory activity.

Question 1 was answered performing the structural characterisation of four galactomannans from three different origins: a) coffee infusion and coffee residue galactomannans presenting immunostimulatory activity; b) locust bean gum (LBG) galactomannan not presenting immunostimulatory activity; and c) *Aloe vera* acetylated galactomannan presenting immunostimulatory activity. These polysaccharides were submitted to sugar and glycosidic-linkage analyses and were hydrolysed by an *endo*-β-D-(1→4)-manannase. The oligosaccharide fragments were also analysed by electrospray-ionization mass spectrometry and tandem mass spectrometry.

Coffee infusion, coffee residue, and *Aloe vera* galactomannans presented similar degree of branching and molecular weight whereas LBG galactomannans presented higher degree of branching and molecular weight. All galactomannans present arabinose residues as branching side chains. The acetylation degree of LBG galactomannans approached zero whereas *Aloe vera* galactomannans presented an acetylation degree of 2.08, coffee residue galactomannans presented 0.98, and coffee infusion galactomannans presented an acetylation degree of 0.08. The location of the acetyl groups was irregular in all polymers. The results obtained allowed to infer that low branching, short chains and some acetylation seem to promote the immunostimulatory activity attributed to the galactomannans.

To answer to question 2, a methodology using the roasting of the residue at 160 ºC and at 220 ºC and its extraction with hot water and 4 M NaOH solutions at 20, 60, and 120 ºC was tested. The roast of the residue at 160 ºC and the sequential extraction allowed the extraction of 56% of the coffee residue galactomannans and, simultaneously, 54% of the arabinogalactans. The galactomannans kept their characteristic structure of acetylated polysaccharide composed by a backbone of β-(1→4)-linked mannose residues and Gal and Ara residues as side chains. At 220 ºC, the galactomannans were partially degraded and the yield of extraction was much lower than at 160 ºC. Nevertheless, even at this temperature, the galactomannans presented acetylated residues as well as pentose residues as side chains. These observations allowed to infer that these polysaccharides are highly resistant to high temperatures and alkali reagent conditions.

In order to better understand the thermal stability of coffee residue galactomannans and the influence of the presence of arabinogalactans in galactomannans stability, a thermogravimetric analysis was performed using the coffee residue polysaccharides as well as structural related polysaccharides such as cellulose, LBG galactomannan and gum Arabic, an arabinogalactan. The galactomannans are thermally stable during 3 h at 200 ºC, whereas the arabinogalactans are stable at 180 ºC. According to the thermogravimetric profiles obtained, and by the calculation of the activation energy of thermal decomposition, the coffee residue presented a lower thermal stability then the galactomanans, possibly due to the presence of the arabinogalactans. Although no changes in the coffee galactomannan thermogram were observed upon a thermal treatment at 200 ºC during 3 h have been observed, structural changes involving the formation of new glycosidic linkages, namely, the formation of mannose residues O-2 and O-6 linked, transglycosilation reactions, depolymerization, formation of anhydrohexose residues on the reducing end, and mannose-glucose isomerization have been observed. These changes promoted galactomannan solubilisation in cold water.

The results obtained allow to propose that coffee residue can be submitted to a roasting followed by an hot alkali extraction to obtain galactomannans in high yield. These polysaccharides can be soluble in cold water upon thermal treatment at 200 ºC, allowing their use in food formulations, namely, by preparation of acetylated compounds, low branched and with short chains in order to promote their immunostimulatory activity.

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ÍNDICE

CHAPTER I

INTRODUCTION

I.1. POLYSACCHARIDES WITH IMMUNOSTIMULATORY ACTIVITY

Polysaccharides are widely distributed in nature: they can be found in plants, animals and microorganisms, performing different biological activities. In recent years some bioactive polysaccharides isolated from natural sources have appealed attention in the biochemistry and pharmacology area. A large range of polysaccharides have been shown to have biological activities, namely, mannan from yeast showing anti-tumour (Suzuki et al., 1969), arabinogalactan from seeds of *Centrosema pubescens* presenting immunological activities (Silva et al., 2000), xyloglucans from plant presenting immunostimulatory activities (Wagner, 1990), pectic polysaccharides isolated from Chinese herbs presenting anticomplement activity (Yamada, 1994), (1→3)-β-glucan from the fungi *Dictyophora indusiata fisch* presenting anti-inflammatory activity (Hara et al., 1982), galactomannans from seeds of *Leucaena* sp. and *Medicago sativa* and the seed hulls of *Glycine max* presenting anticoagulant and fibrinogenic activities (Hussein et al., 1998).

Lipopolysaccharides (LPS) are endotoxins and the major components of the external membrane of Gram-negative bacteria. The LPS comprise three distinct structural domains constituted by an O-antigenic chain (O-chain), an oligosaccharide core, and a fatty acid-rich endotoxin moiety, the lipid A. The LPS have immunostimulatory activity (Alving, 1993) as well as they promote a wide variety of pathophysiological effects (Anspach, 2001; Ogikubo et al., 2004). They constitute a very active and multivalent molecule of bacterial origin, and the comparison with other phosphoglycolipids showed that the constitution of lipid A is unique. Lipid A adopts a peculiar conformation that is responsible for its immuno activities. Rietschel et al. (1994) described the LPS capacity to affect immune cells.

The majority of the assays to evaluate the immunostimulatory activity of polysaccharides use LPS as positive control and Phosphate Buffered Saline (PBS) as a negative control. As polysaccharides are usually recovered in laboratories that are not required to fulfil aseptic conditions, they can be contaminated with LPS. This is why it is important to remove these compounds out of the samples. To reduce endotoxin contamination, ion-exchange chromatography, affinity adsorbents, gel filtration chromatography, ultrafiltration, or sucrose gradient centrifugation have been proposed (Magalhães et al., 2007). Also, the application of Triton X-114 phase partitioning is a

nondegradative method that was also proposed for removal of LPS from polysaccharides (Adam et al., 1995). However, depending on the samples, these techniques may not be so efficient (Magalhães et al., 2007). Polymyxin B has also been used for removal of any contaminant bacterial LPS from pectic polysaccharides (Dourado et al., 2004) as polymyxin B binds to the lipid A region of LPS (Morrison & Jacobs, 1976). Polymyxin B is a small peptide derived from *Bacillus polymyxa* that has widespread antibiotic activity against Gram-negative bacteria. The immobilization of this antibiotic into a gel medium forming an immobilized-polymyxin B complex is usually used (Magalhães et al., 2007). The efficiency of this procedure can be confirmed by the E-toxate (*Limulus Amebocyte Lysate*) test. The E-toxate reagent is prepared from a lysate of the circulating amebocytes of the horseshoe crab, *Limulus polyphemus*. When exposed to infinitesimal quantities of LPS, the lysate increases in opacity as well as viscosity and may form a gel, depending on the concentration of LPS (Reinhold & Fine, 1971). Thus, the immunostimulatory activity assays of polysaccharides should include a detoxifying step in order to state that the activity is due to the samples under study and not by the presence of contaminating LPS. Few studies have, however, reported the use of an approach to remove the possible presence of LPS when evaluating the immunostimulatory activity of polysaccharides. Among these are the studies of the *Acanthopanax senticosus* polysaccharides, although their composition has not been reported (Han et al., 2003), the arabinan-rich pectic polysaccharides (Dourado et al., 2004), and the galactomannans from coffee, *Aloe vera* and locust bean gum (Simões et al., 2009).

Despite the biological activity of polysaccharides is known for years, the lack of structural details limits the study of their potential applications. The different chemical structures influence the various biological activities (Yang & Zhang, 2009). Their properties and applications are dependent on their detailed structure, namely, their composition in monosaccharides, type of linkages, branching, and anomeric configurations (Tzianabos, 2000). Also, the effect of the conformation and the polarity of the polysaccharides are reported to modulate their biological activity (Leung et al., 2006). It becomes increasingly important to understand the detailed structure of the bioactive compounds to better understand their function. The study of immunostimulatory active polysaccharides must involve complementary fields in chemistry and life sciences, the reason why only a few studies on structure-activity of polysaccharides has been found in

literature. The majority of the studies only state one part, usually the biological effect, not relating that with the structure of the polysaccharide, as for example occurs in the study developed in a polysaccharide from of a Chinese herbal composed by a glucan and an arabinogalactan that presented *in vitro* immunomodulatory activities shown by cell proliferation and macrophages and T cells promotion (Yang et al., 2006), or the galactomannans isolated from *Gleditsia macracantha Desf.* seeds that presented hypocholesterinemic and hypoglycemic activities (Rakhmanberdyeva et al., 2005). Another drawback of also a large number of studies is the presence of bioactive compounds beyond the polysaccharides that may mislead the conclusions, as shown by Monobe et al. (2008) when demonstrating the immunostimulating activity of a high molecular weight pectin from tea polysaccharides. Another example where the impurity of the samples could be an outcome, leaving doubts which is the specific component responsible for a specific activity, is the different compounds of the extracts from low-grade green coffee and spent coffee that could presented different activities. The phenolics, chlorogenic acids and brown pigments presented anti-tumour, anti-allergenic, and antioxidants activities (Ramalakshmi et al., 2009). This is one example that other components besides polysaccharides could be responsible for the different activities.

A constant debate occurs as to the origin of the immunostimulation effects of β-(1→3)-glucans, and rationalization of these effects is complicated by the size of the glucans, as well as by their shape and dynamic supramolecular organization in aqueous solutions (Sletmoen & Stokke, 2008). The detectable interactions between dectin-1, a major β-glucan receptor on macrophages, and the positive biological effects, required at least 10 or 11 glucose residues (Palma et al., 2006). Dectin-1 is highly specific for β-(1-3)-glucans from fungi but it cannot recognize equally all families of this homopolymer. As such, dectin-1 differentiates glucan ligands depending on their structural parameters such as length of the polysaccharides, and more importantly, side-chain branching. Approaches have been proposed for obtaining well-defined glucans and also for determining the minimal chain length and branching required for optimal activity (Adams et al., 2008), although no conclusive remarks have been yet obtained.

Acemannans from *Aloe vera* presented some of the health benefit activities that are attributed to this plant (Hamman, 2008). The *Aloe vera* extracts have shown the promotion

of wound healing (Davis et al., 1994), skin hydration (Dal´Belo et al., 2006), anti-inflammatory (Davis et al., 1994), antifungal (Rodrígues et al., 2005), antidiabetic (Yongchaiyudha et al., 1996), anticancer (Steenkamp & Stewart, 2007), immunostimulatory (Chow et al., 2005, Simões et al., 2009), and gastro protective (Yusuf et al., 2004) properties. Simões et al. (2009) showed that a commercial sample of *Aloe vera* acemannan stimulated murine B- and T-lymphocytes, as evaluated by the *in vitro* expression of the surface lymphocyte activation marker CD69, more marked on B- than on T-lymphocytes. *Aloe vera* acemannan can interact with the mannose receptors present on several cells, including macrophages, stimulating the immune response (Leung et al., 2006). Acemannan is the designation for the acetylated mannan from *Aloe vera* commercially available. The *Aloe vera* acemannans are composed by a backbone of acetylated β-(1→4)-mannose residues with α-(1→6)-galactose residues as single side chains (Reynolds, 1985). The acetylation occurs at the C-2, C-3 and C-6 of mannose residues with an acetyl/mannose ratio of approximately 1:1, and the mannan backbone contains interspersed β-(1→4)-linked glucose residues (Fogleman et al., 1992; Manna & McAnalley, 1993; McAnalley et al., 1999; Talmadge et al., 2004; Hamman, 2008). Although Ni et al. (2004) reported that the acemannan found in *Aloe vera* has an unique structure among other well-known plant mannans, more recently, a similar structure has been also identified in coffee (Nunes et al., 2005). Little is known about the pattern of acetylation of these compounds, in particular the distribution profile of acetyl groups along the polymeric chain, as well as lack of establish relations between structure and function.

Galactomannans from coffee have also been shown to have beneficial biological activities, namely, fermentation in the human colon and thereby contributing to the physiological effects generally associated with fiber fermentation (Gniechwitz et al., 2007; Reichardt et al., 2009) and immunostimulatory activity by stimulated murine B- and T-lymphocytes, as evaluated by the *in vitro* expression of the surface lymphocyte activation marker CD69, more marked on B- than on T-lymphocytes (Simões et al., 2009). As galactomannans, the arabinogalactans also presented *in vitro* immunostimulatory properties (Gotoda et al., 2006). Dourado et al. (2004) extracted an arabinan-rich pectic polysaccharide from *Prunus dulcis* seeds that induced stimulatory effect, as evaluated by the *in vitro* and *in vivo* expression of lymphocyte activation markers and cells culture proliferation. The detailed structure of this immunostimulatory active arabinan-rich pectic

polysaccharide was established by the ration 5:2:1 for arabinose, uronic acids and xylose, respectively, with an arabinose linkage composition of 3:2:1:1 for T-Ara*f*:(1→5)-Ara*f*:(1→3,5)-Ara*f*:(1→2,3,5)-Ara*f*, respectively.

I.2. COFFEE RESIDUE

The agro-food industry and the agricultural production provide large volumes of solid wastes and residues, produced either by secondary processing industries or in the agro-forestry sector. The majority of these materials become a source of environmental problems. However, if residues are utilized, they are not considered as wastes but new resources. Some examples of residues that can be recovered and upgraded are corn cobs, cereals straw, vine pruning, various grasses and reed stems, cotton stalks, sugarcane and tequila bagasse, banana and coconut residues, corn husks, coffee pulp, coffee husk and coffee residue, cotton seed and sunflower seed hulls, peanut shells, rice husks, sunflower seed hulls, waste paper, wood sawdust and chips (John et al., 2007; Philippoussis & Diamantopoulou, 2011).

Many technologies involving chemical or biological processes are adopted to reduce the pollution, renewable raw materials, and promotes value added products by the use of agricultural and industry wastes and residues (Webb et al., 2004). Polysaccharides could be found as wastes from vegetable industrial processing, and the search for a sustainable production of these polysaccharides in order to reutilize them, passes through the implementation of processes and technologies that are safe for the environment (Poli et al., 2011),

Starch, pectins, and cellulose can be applied in medical areas as an inert diluent for drugs, wound dressing, and could be applied as constituents for scaffolds and implants in tissue engineering. Pectins, celluloses and hemicelluloses can be used as dietary fibres, affecting positively the health, as well as employed in the industrial food production as emulsifiers, additives, thickeners, gelling and texturizing agents (Sanjay & Gross, 2001). Pectin, from citrus or apple fruits, is widely for the stabilisation of acidified milk drinks and yogurts and used as functional food ingredient, being its industrial utilisation based on

its gelling properties, used for example in the production of confectionery products, fruit juice, jellies and jams (Willats et al., 2006).

Starch from potato, corn and other starchy plants, is the main source of sweeteners such as maltose, glucose or fructose syrups in addition to other products including prebiotics, mannose, sorbitol and ascorbic acid, ethanol, etc. Inulin and related fructooligosacharides, from chicory, are used as functional food ingredients and can contribute to reduce the risk of many diseases by stimulating the immune system (Kaur & Gupta, 2002). Starch is studied to be applied as thermoplastic starch and for the processing of foamed materials for loose-fill packaging (Gandini, 2008).

Concerning coffee waste and by-products, depending upon the method of coffee cherries processing, wet or dry, the solid by-products obtained are designated as pulp or husk, respectively (Pandey et al., 2000). In many of the coffee producing countries the disposal of coffee pulp causes significant environment problems and assigning new uses for this agro-industrial waste is gaining more attention. Coffee pulp is rich in carbohydrates, especially in pectin. One of the popular uses of coffee pulp is for mushroom cultivation substituting the commercially popular wheat straw substrate by one agro-industrial waste- coffee pulp (Salmones et al., 2005). Coffee husk is a lignocellulosic material that could be used for the aroma production, where, under optimized conditions, a total of 13 compounds were produced which included alcohols (2), aldehyde (1), ketones (2) and esters (8). Ethyl acetate was the prominent compound, followed by ethanol (Soares et al., 2000). The main coffee industry residues are coffee silverskin and spent coffee grounds. Coffee silverskin is the coffee bean coat obtained as a roasting process by-product, while spent coffee grounds is a residue with fine particle size with high humidity (80 to 85%) obtained during the treatment of raw coffee powder with hot water or during the industrial instant coffee preparation. Almost 50% of the worldwide coffee production is processed for soluble coffee preparation (Ramalakshmi et al., 2009), therefore, spent coffee grounds are formed in large amounts, with a worldwide annual generation of 6 million tons (Tokimoto et al., 2005). Coffee silverskin has been used as soil fertilizer or as fuel (Saenger et al., 2001) but most of these residues remain unutilized, being discharged to the environment or burned for elimination, which are not environmentally friendly techniques and is a waste of these materials.

Coffee residue is a by-product resultant from coffee beverage preparation, composed by the materials that were not able to be extracted by the hot water. Some attempts of reutilisation of coffee residue have been made, using as an animal feed, although for ruminants the very low digestible energy and negative metabolisable energy contents indicate that coffee residue are worthless as feed (Givens & Barber, 1986). Also, it is used to produce biodiesel by extraction and transesterification of the oil. The deffated coffee residue is a suitable material for garden fertilizer, feedstock for ethanol, and as fuel pellets (Kondamudi et al., 2008), as an antioxidant material due to the antioxidant compounds that remained in the residue (Yen et al., 2005), and as substrate for production of edible mushrooms (Leifa et al., 2001). Furthermore, coffee residue can be also used for preparation of activated carbon and for application as adsorbent for dyes removal in wastewater (Hirata et al., 2002). To our knowledge, none of these strategies have yet been routinely implemented. In some cases, it is used as fuel in industrial boilers of the own industry due to its high calorific power of 5 Mcal/kg (Silva et al., 1998).

The main roasted coffee bean components are caffeine, polysaccharides, chlorogenic acids, lipids, other nitrogenous compounds, volatiles, and melanoidins. The polysaccharides comprise between 30–43% (Redgwell et al., 2002a) of coffee bean dry weight, and are composed mainly by galactomannans, type II arabinogalactans, and cellulose. During the preparation of coffee brew, a part of these polysaccharides, mainly galactomannans and also arabinogalactans, are extracted (Nunes & Coimbra, 2001). However, the majority of the galactomannans, as well as the arabinogalactans, remain associated to the cellulose-rich cell wall matrix.

The galactomannans are the main components of coffee by-product (Simões et al., 2009). Coffee galactomannans (Fig. 1.1) are composed by a linear β-(1→4)-linked D-mannopyranose residues backbone substituted at O-6 with single residues of α-D-galactopyranose residues (McCleary et al., 1985). The galactomannans are acetylated polysaccharides (Oosterveld et al., 2004), as acetyl groups have been observed at the O-2 or O-3 of mannose residues (Nunes et al., 2005). Single acetylated mannosel residues, di-acetylated mannose residues and consecutively acetylated mannose residues are present in coffee galactomannans. Also, coffee galactomannans are composed by single arabinose residues as side chains and contain β-(1→4)-linked glucopiranose residues interspersed in

the main backbone (Nunes et al., 2005). This structure identified in coffee has been also identified in galactomannans from different sources, such as *Aloe vera* (Gowda et al., 1979) and the seeds of *Gleditsia ferox Desf.* (Egorov et al., 2003), *Cassia grandis* (Joshi & Kapoor, 2003), *Cassia javahikai* (Singh et al., 2009a), *Cassia pleurocarpa* (Singh et al., 2009b), *Dimorphandra gardneriana Tul.* (Cunha et al., 2009), *Adenanthera pavonina, Caesalpinia pulcherrima, Gleditsia triacanthos, Sophora japonica* (Cerqueira et al., 2009), and *Caesalpinioideae* and *Faboideae* subfamilies (Pollard et al., 2010). All these galactomannans present side chains randomly distributed where the segments of the chain composed by unsubstituted β-D-mannopyranose units are interspersed with chain segments in which α-D-galactopyranose side chains are linked to one mannose unit of the main chain (Daas et al., 2000). They differ also in their degree of polymerization (number of mannose residues constituent of the main backbone) and degree of branching (ratio of mannose to galactose residues) (Robinson et al., 1982). Beyond the origin, the degree of polymerization as well as the Man/Gal ratio of galactomannans depends on the plant age, the growth conditions, and even the method of extraction of the polysaccharides (Kok, 2007). Although the structural similarity has been verified in all plant seed galactomannans, the galactomannan of the seeds of *Retama raetam*, a wild plant belonging to the *Fabaceae* family, presents an unusual backbone structure, containing (1→3)-linked residues together with a small proportion of β-(1→4)-linked D-mannopyranose residues with galactopyranose units attached at O-6 (Ishurd et al., 2004). This shows that each species should be analyzed for its own specificities and structural features

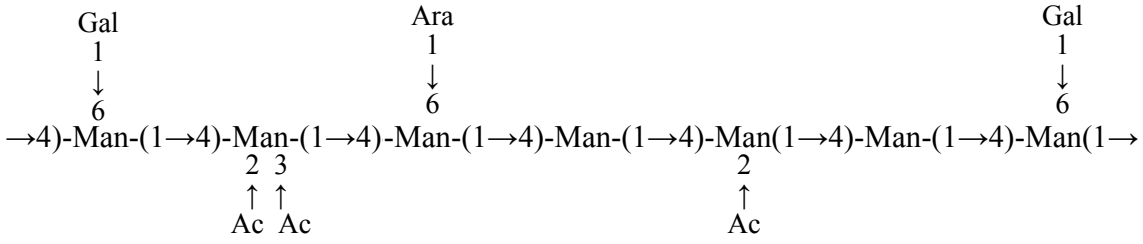


Fig. 1.1 *Scheme of a galactomannan from a roasted coffee infusion.*

Coffee type II arabinogalactans (Fig. 1.2) are polysaccharides usually covalently linked to proteins. They are composed by a main backbone of (1→3)-linked β-D-galactose residues, some of them substituted at the O-6 position with short chains of (1→6)-linked β-D-galactose residues (Bradbury, 1990). The arabinogalactans are heterogeneous both with regard to the degree of branching and the degree of polymerisation of their arabinan side-chains (Fischer et al., 2001).The galactose residues of these (1→6)-linked β-D-galactose side chains can be substituted at the O-3 position with single α-arabinose residues and (1→5)-linked arabinose disaccharides. Terminally linked to these (1→6)-linked β-D-galactose side chains can be glucuronic acid residues (Redgwell et al., 2002a). In some hot water soluble type II arabinogalactans, rhamnoarabinose and rhamnoarabinoarabinose side chains are also linked to the O-3 position of the (1→6)-linked β-D-galactose side chains substituted at intervals in the O-6 position with various combinations of arabinose and galactose residues (Bradbury, 2001). Arabinogalactans have a β-(1→3)-Gal*p*/β-(1→ 3,6)-Gal*p* ratio of 0.80, containing 2 mol% of glucuronic acid residues, present single α-L-Ara*f* residues and [α-L-Ara*f*-(1→5)-α-L-Ara*f*-(1→] disaccharide residues as side chains (Nunes et al., 2008). More than 10% of glucuronose residues occurred as non-reducing terminal units on (1→6)-linked galactose side chains (Redgwell et al., 2002a). These arabinogalactans also present side chains at O-3 position of the β-(1→6)-linked galactopyranose residues composed by [α-L-Rha*p*-(1→5)- α-L-Ara*f*-(1→] and [α-L-Rha*p*-(1→5)- α-L-Ara*f*-(1→ 5)- α-L-Ara*f*-(1→] oligosaccharides (Nunes et al., 2008).

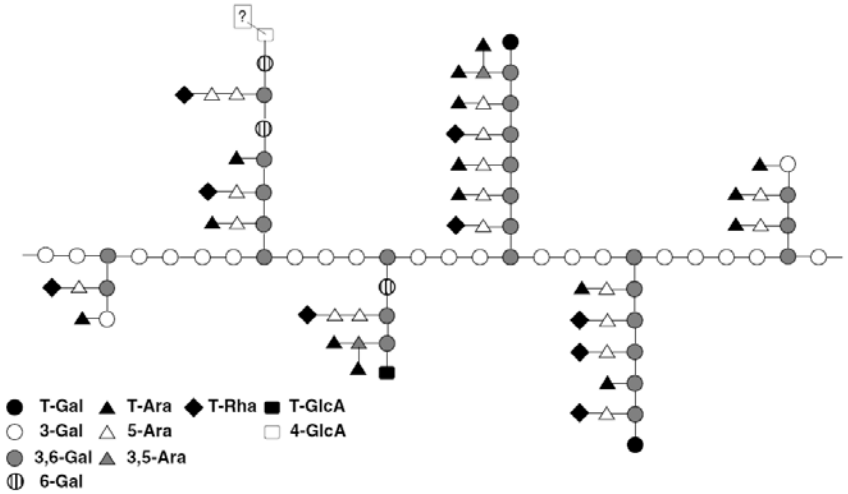


Fig 1.2 *Structural arrangements for arabinogalactan-protein of green coffee (Nunes et al., 2008)*

I.2.1. MODIFICATIONS OF POLYSACCHARIDES STRUCTURE BY ROASTING

Coffee roasting process is a fundamental unit operation in coffee production, where the green coffee beans are submitted for a certain period of time to a dry heat treatment, which is atypically high temperature for a food product. Physical and chemical changes occur (Belitz & Grosch, 1987) followed by the formation of various types of aroma and flavour compounds (Maria et al., 1994). Changes in colour of the coffee bean happen and the polysaccharide content turns more extractable (Trugo, 1985). The roasting occurs in roasters where hot air roast the coffee beans, normally between 180 °C to 230 ºC, during 12 to 15 minutes (Smith, 1985), depending on the degree of roasting required. The roasting process can be divided in 3 steps. In the first step the water is removed from green coffee beans. After that, a series of pyrolytic reactions occur in coffee beans, resulting in the CO_2 released and the formation of new substances contributing to its unique flavour and taste. In the third step, a rapid cooling of the roasted coffee beans is performed (Illy & Vianni, 1995).

The roasting degree is qualitatively evaluated by the colour of coffee beans, resulting in a simple classification as light, medium, or dark roast coffees. The roasting degree can also be quantified by the correspondent dry or organic weight loss. A light roast shows about 3-5% loss, medium 5-8%, and dark 8-14%, together with the humidity that the green beans contains (Clarke, 1985).

Although coffee bean galactomannans are known to be more resistant than arabinogalactans (Oosterveld et al., 2003), it has been shown that during the roasting of the coffee beans, the arabinogalactans and galactomannans undergo several structural modifications. Galactomannans can undergo depolymerization, debranching, Maillard reactions, caramelization, isomerization, oxidation, decarboxylation, and melanoidins formation (Nunes et al., 2006a). Melanoidins are thermally transformed galactomannans, composed by a heterogeneous high molecular weight material, brown in colour and containing nitrogen (Nunes & Coimbra, 2007; 2010). Structural characterization of coffee melanoidins has shown that polysaccharides, proteins, and chlorogenic acids are involved in coffee melanoidin formation (Nunes et al., 2005). Maillard reaction, caramelization, oxidation, and decarboxylation pathways were identified by detection of Amadori

compounds, anhydromannose, mannonic acid and arabinonic acid, among other modifications in the reducing end (Nunes et al., 2006a).

One of the main changes during coffee roasting is the increase in extractability of coffee galactomannans (Nunes & Coimbra, 2001). The higher the degree of roast, the higher the amount of galactomannans present in the coffee brews of both *Coffea arabica* (Nunes & Coimbra, 2002a) and *Coffea canefora* (robusta) (Nunes & Coimbra, 2002b). Also, the roasting of the coffee bean promotes the easy of extraction of cell wall polysaccharides with NaOH aqueous solutions (Oosterveld et al., 2003). Different amounts of galactomannans from coffee beans can be extracted with hot water, depending of the origin of the coffee as well as their degree of roast. Arabica coffee infusions contain higher amount of galactomannans (62-80%) than Robusta (44-67%) and the amount of galactomannans in solution increases with the roasting of the coffee (Nunes & Coimbra, 2001; 2002a; 2002b). The roasting of coffee beans promotes an increase of the bean volume and the appearance of larger cell wall micropores due to the opening the cell wall matrix (Schenker et al., 2000). These changes also contributed to the increase of the amount of hot-water-soluble polysaccharides, namely, the galactomannans, when the ground roasted coffee beans are used to prepare the coffee brew (Nunes & Coimbra, 2001). Roasted coffee beans are composed, depending of the degree of roast, by 30-43% of polysaccharides (Redgwell et al., 2002a), being mannans the least degraded polysaccharides, accounting for nearly 50% of total polysaccharides, even for the darkest roasted coffees.

In contrast to cellulose, that is the polysaccharide less affected by the roasting process, coffee arabinogalactans are the most vulnerable of the coffee polysaccharides to degradation during roasting, especially the more labile arabinose residues (Nunes & Coimbra, 2002a; 2002b; Redgwell et al., 2002b) present as side chains. Also, the arabinogalactans depolymerize during roasting and a huge decrease in molecular weight is observed even at after a light roast (Redgwell et al., 2002b). The debranching of the arabinose side chains seems to occur more rapidly than the hydrolysis of the galactan backbone (Ooosterveld et al., 2003).

I.2.2. EXTRACTABILITY OF POLYSACCHARIDES FROM CELLULOSE MATRICES

The extractions of polysaccharides from cell wall matrices are based on the exploitation of the particular properties of each polymer and should avoid degradation and structural alteration. Also, the extractions are affected by various factors, such as the temperature of extraction, the time of extraction, and the solvent-solid ratio. In general, hot-water extraction is the wider method used for polysaccharide extraction. However, hot-water extraction of polysaccharides is associated with low yields, that may increase using longer extraction times and high temperatures. In some cases ultrasound or microwave assisted extractions can accelerate the extraction process and thus improve the extraction of polysaccharides. Hot water extractions are related to the cooking process, ruling the textural quality of the polymers and the depolymerization of polysaccharides, leading to tissue softening and polymer solubilisation.

The procedures with chelating agents are used to extract pectic polysaccharides and could involve for example such as trans-1,2-diaminocyclohexane N,N,N´,N´-tetraacetic acid (CDTA) to disrupt plants cell wall calcium bridges, facilitating the removal of the pectic polysaccharides (Coimbra et al., 1994).

In leguminous plants the majority of the cell wall polysaccharides is only extracted with high alkali concentrations due to the strong association between the polysaccharides. Polymers with chains associated by hydrogen bonds could be extracted with alkali solutions, generally NaOH or KOH. The higher the hydrogen bonding, the higher the concentration of alkali solution that needs to be used, varying between 0.5 and 8 M. The ester linkages, which often bind polysaccharides with other organic molecules, namely, phenolic compounds, are easily destroyed by alkaline treatments, normally by less concentrated alkali solutions. These solutions should contain $NaBH_4$ and O_2–free solutions to prevent peeling degradation reactions.

Hot alkali extractions can improve the extractability of polysaccharides (Miyajima et al., 2001). As occurred with the extractions with hot water, the high temperatures promote structure softening, helping the polysaccharides extraction. Treatments with alkali (Hitchcock, 1977) and heat (Handa & Tominaga, 1969) often have been applied to extract

water-soluble polysaccharides adsorbed on natural particulate matter with controlled changes of polymer structure. Extractions with hot alkali did not noticeably change the microfibrillar network (Reid & Garcia, 1976). If the aim is to reach as high molecular weight as possible, only cold extraction could be used (Hoije et al., 2005). Hot alkali extractions have also been shown to dissolve more arabinoxylans (Hoije et al., 2005) as well as to increase substantially the extractability of lignin and cellulose (Vilpponen et al., 1993; Pan et al., 2005).

I.3. DETERMINATION OF POLYSACCHARIDES STRUCTURE BY MASS SPECTROMETRY

Mass spectrometry has become an useful technique with diverse applications in chemical and biological fields (Sleno et al., 2004) and is today one of the most sensitive methods for the structural characterization of biomolecules (Aielo et al., 2011).

I.3.1. BASICS OF MASS SPECTROMETRY

A mass spectrometer is an instrument that involves three general stages: sample ionization; separation on the analyser according to mass/charge ratio (*m/z*); and detection of the ions formed. In the ionization sources, the samples molecules are ionized, and ions are produced in the gas phase. Most common ionization sources are Eletrospray Ionization (ESI), Matrix Assisted Laser Desorption Ionization (MALDI) and Electron Impact (EI). In this thesis, in order to obtain detailed structural information of polysaccharides, the most common ionization techniques of polysaccharides analysis by mass spectrometry used are electrospray ionization (ESI), and matrix-assisted laser desorption ionization (MALDI). EI was used in the gas-chromatography analysis, since the mass spectrometer coupled to the GC apparatus has an EI source. ESI and MALDI are known as soft ionization technique as the sample is ionised and the ions formed possessed low internal energy, not promoting fragmentation in source. This is an advantage since allow to identify the molecular weight of all the molecules presented in the samples. Both ESI and MALDI ionization sources are analytical techniques very sensitive, being the analyte concentrations being able to be analysed as low as picomolar range. ESI was used to study oligosaccharides with molecular weight lower than 2000 Da. MALDI had the disadvantage of not allowing the

analysis of low molecular weight OS (> 700 Da) due to the presence of ions formed due to the ionization of the matrix. Analysis of higher molecular weight oligosaccharides are usually carried out by MALDI-MS. The biggest difference between MALDI and ESI is the phase of the sample introduced into the ion source: ESI uses a dissolved sample, whereas MALDI uses the solid state. The ESI is capable of reproducing data better than MALDI and both the ESI-MS or MALDI-MS spectra are simple and easily to understood. These methods of ESI and MALDI ionization have been applied in the analysis of mixtures of polymers and, more specifically, in the analysis of oligosaccharides mixtures (Zaia, 2004; Bauer, 2012). Due to the high affinity of polysaccharides for alkali metals, and due to the fragmentation influence among other factors by the type of precursor ion formed, the correspondent OS were mainly detected as sodium adducts ($[M+Na]^+$) (Zaia, 2004), and in some cases detected as $[M+Li]^+$, depending on the cases that different metal coordinates oligosaccharides for MS^n analysis could take advantages. Also protonated ($[M+H]^+$) could be detected when the concentration of H^+ is higher or in the case of a nitrogen is presented in the OS structure, namely due to the presence of hexosamines.

To separate the generated ions, different types of analysers could be used: quadrupole (Q), ion trap (IT), time-of-flight (TOF). They can be used alone, as in the case of GC-MS where one Q analyser is connected to the EI source, or in the IT that is connected to the ESI source. However analyser can also be combined in the same mass spectrometer in order to increase sensitivity or to allow MS/MS analysis to be performed, such as quadruple-orthogonal acceleration time-of-flight (QTOF) or the triple quadrupole (QqQ), both usually connected with an electrospray source. MALDI source is usually connected to the TOF analysers. The mass analyser is the part of the mass spectrometer in which the ions formed in the source are separated according to their *m/z* values. Similar to the ionization process, in terms of the available methodologies, there are numerous systems that can isolate ions based on their *m/z* values. These analysers differ in terms of mass range, resolution, and capacity to perform tandem mass spectrometry (MS/MS and MS^n). The analyser TOF is the simplest analyser present in mass spectrometry where the separation of ions involves measure of the time that the ions take to go through the flight tube: from the ionization source until it reaches the detector. The ions have different mass values, and different ions group will be formed due to different velocities along the flight tube. To increase resolution and the length of path, TOF analysers usually have reflectrons

that allow to obtain mass spectra with high resolution. IT analysers is based on physical principles similar to the quadrupole analyser but tridimensional.It consists in capturate the ions into a box by the electric field application and them eject selectively the ions in accordance to the *m/z* range (Johnstone et al., 1996). The advantages of the IT mass spectrometer include compact size, and the ability to trap and accumulate ions to increase the signal-to-noise ratio of a measurement. Though IT is capable of multiple mass spectrometric experiments (MS^n), Ion trap mass spectrometers coupled with either ESI or MALDI are a very useful technique for the structural characterization of carbohydrates. This utility comes from the ion trap´s capacity to perform multiples stages of mass spectrometry (MS^n) (Fernández, 2007). In the last stage of an MS experiment, the ions are measured in the detector and the signal was processed and exhibited as a mass spectrum.

I.3.1. FRAGMENTATION PATTERN OF CARBOHYDRATES

The study of the mass spectra allows the identification of oligosaccharides molecular weight. This information, when combined with the sugar composition and methylation analysis allows to propose a number of possible structures for the OS in study. However, detailed information of the sequence of OS and sureness on the identification of these structures is only accomplished by tandem mass spectrometry analysis (MS/MS). MS/MS consist on the isolation of a selected ion, called a precursor ion, that is then fragmented and the resulting product ions are further analysed. Fragmentation of the precursor ions occurs mainly in the glycosidic linkage even though some cross-ring fragmentations can be observed thus generating valuable structural information like sequence of monosaccharides and type of branching (Zaia, 2004). Differentiation of isomers can also be achieved. In one-stage mass spectrometry, ions formed in the ion source are separated in the mass analyser before reaching the detector, and in tandem mass spectrometry, MS/MS experiment, for example in a triple quadruple or in a Q-TOF mass spectrometers, a specific ion is selected (precursor ion) in the first mass analyser (MS^1) and then submitted to a fragmentation in the collision cell and finally on the second analyser (MS^2) the fragments formed (fragment ions) are separated and analysed on the detector (Fig. 1.3).

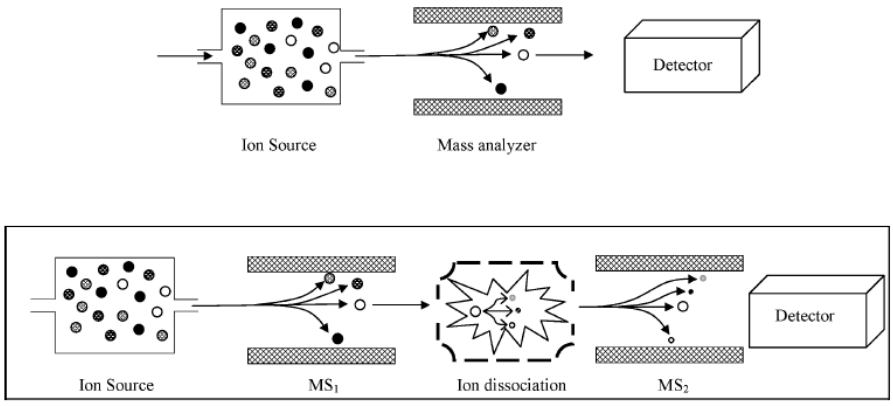


Fig. 1.3 *Comparison of one-stage mass spectrometry (top) and tandem mass spectrometry (bottom).*

Tandem mass spectrometry (MS/MS) is an useful tool to improve the polymer structural knowledge. In a simplistic way, it can be understood as the coupling of *n* stages of mass analysis. The MS/MS spectrum gives important information about the polymer structure. It could be done by coupling of more stages of mass spectrometry analysis (MS^n), being these described MS/MS experiment, consecutively repeated. In figure 1.3 it was shown that in tandem mass spectrometry, a specific ion is selected in the first mass analyser (MS^1) and then subjected to collision in the collision cell, whereas the second analyser (MS^2) separates the product ions before they reach the detector.

Mass spectrometry is a powerful and highly sensitive analytical tool allowing structural characterization of oligo and polysaccharides. MS has been successfully used for the study of the structural details of oligo and polysaccharides, namely using soft ionization methods such as electrospray, and tandem mass spectrometry (ESI-MS^n), due to having the advantage of using underivatized oligomers, even when present in mixtures and with low abundance.

The analysis of polysaccharides in their native form is difficult due to their high molecular weight, thus, for MS polysaccharides analysis, they must be firstly converted into oligosaccharides (OS) by partial acid or enzymatic hydrolysis in order to obtain lower molecular weight oligosaccharides more easily analysed by MS. The hydrolyzate obtained is then fractionated by size exclusion, and selected fractions are analysed by ESI-MS The enzymatic degradation has the advantage of being specific to one specific polymer and can be applied to a mixture of polysaccharides eliminating the need for purification, which

often removes modifications (e.g. acetylation). Enzymes are also specific for certain linkages and cleavage sites. The information obtained can be useful in structure elucidation. The most important part of the analysis of the OS is finding MS fragments that are specific for one structure and allow the alternatives elimination.

ESI-MS gives information about the molecular weight of OS even when present in mixtures and, in combination with the sugar residues composition, allows the proposal of a range of structures (Zaia, 2004). The characterization of oligosaccharides can be obtained by MS, through the determination of the molecular weight (ESI-MS and MALDI-MS spectra), but also by fragmentation of oligosaccharide by Tandem mass spectrometry (MS/MS). MS/MS proved to be a valuable tool in the structural characterization of carbohydrates because detailed information about their structure like: identification of the monosaccharide units; determination of the anomeric features; determination of the monosaccharide sequence; linkages determination; and identification of modifying groups can be obtained (Asam & Glish, 1997; Zhou et al., 1990). The identification of the fragment ions in the MS / MS spectrum also provides information on the information of the branching pattern (Harvey et al., 1997) and in some cases on the linkage type present in the structure (Bahr et al., 1997; Harvey, 2000). This technique has the advantage over the other commonly used methods due to its feasibility even on complex mixture of oligosaccharides and in trace amount of samples, and is sensitive and high throughput. Since the isomeric structures produce different fragmentation spectra, the analysis of isomeric mixtures is also possible. Also, when mass spectra gives unclear results by the production of two or more precursor ions, doubts can regularly be removed by applying and analysing MS^n (Bauer, 2012).

Domon and Costello (1988) introduced a nomenclature designation for the products resulting from oligosaccharide fragmentation. The nomenclature for oligosaccharide fragmentation used throughout the mass spectrometry field is shown in figure 1.4. Product ions that contain a non-reducing end are labelled with uppercase letters from the beginning of the alphabet (A, B, C), and those that contain the reducing end are labelled with letters from the end of the alphabet (X, Y, Z); subscripts indicate the cleaved ions. The A and X ions are produced by cleavage across the glycosidic ring, and are labelled by assigning each ring bond a number and counting clockwise. The other ions (B, C, Y, Z) result from

glycosidic cleavages. All product ions are labelled with a subscript number identifying the number of sugar residues retained in the fragment ion. (Zaia, 2004)

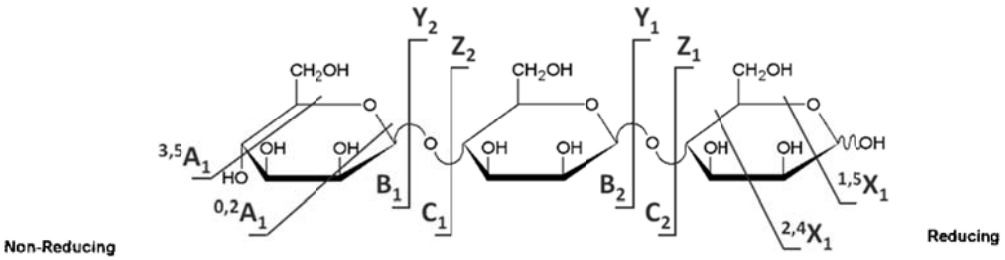


Fig. 1.4 *Nomenclature for oligosaccharide fragment ions according to Domon and Costello(1998).*

The fragmentation pattern of oligosaccharides given by MS/MS spectra resulted mainly of glycosidic cleavages (for example -162 Da, and -180 Da for hexose OS, due to loss of respectively Hes_{res} and Hex unit) between two sugar residues and of cross-ring cleavages with the cleavage of two bonds within the sugar ring (Zhou et al., 1990; Hofmeister et al., 1991). So, OS can generate product ions via glycosidic bond cleavages and cross-ring fragmentation. The cross ring fragmentations observed, allowed to infer the type of linkage present in the disaccharide (Hofmeister et al., 1991; Asam & Glish, 1997). The (1→6) hexose disaccharides fragmented by loss of $C_2H_4O_2$ (-60 Da), $C_3H_6O_3$ (-90 Da), and $C_4H_8O_4$ (-120Da), (1→3) disaccharides by loss of $C_3H_6O_3$ (-90 Da) and (1→2) hexose disaccharides by loss of $C_4H_8O_4$ (-120 Da). Furthermore, the (1→4) linked disaccharides, in addition to the loss of $C_2H_4O_2$ (-60 Da) showed the product ions formed by elimination of $C_4H_8O_4$ (-120 Da), (Hofmeister et al., 1991). An O-2-linked acetyl group would be indicated by an ion of 102 Da (59+43).

I.3.3. APPLICATIONS OF MASS SPECTROMETRY

Besides many papers published about MS were focused in the glycoproteins field, also many papers have come increasingly to be found about oligosaccharide analysis (Harvey, 2000; Chai et al., 2002; Aielo et al., 2011; Tuzimski 2011; Bauer, 2012). As examples, the MS were used to characterize the oligosaccharides from pectins (Ralet et al., 2009), gellans (Gonçalves et al., 2009), carrageenan (Antonopoulosa et al., 2004), and xylo-oligosaccharides from wood (Reis et al., 2004; Reis et al., 2005). Detailed structural features of glucuronoxylans (Reis et al., 2002; 2003), coffee galactomannans and arabinogalactans (Nunes et al., 2005; 2006a; 2008) can also be achieved by mass spectrometry as well as MS could also allow to evaluate the anomeric configuration of the gluco-oligosaccharides (Simões et al., 2007).

ESI-MS/MS spectra of $[M+Li]^+$ and $[M+Na]^+$ ions of reducing glucose disaccharides allowed to identify their anomeric configuration (α or β). These diagnostic differences, based on the differences in the relative abundances of some product ions, were even more relevant considering that no derivatization was needed for obtaining this structural information. A statistical study had shown that anomeric configuration has influence on the combined dependent variables (relative abundances of *m/z* product ions in all the three mass spectrometers used and diagnostic model were built (Simões et al., 2007).

Detailed structural features of glucuronoxylans can also be achieved by ESI-MS and MS/MS analysis, namely, for determination of the main xylose backbone and substitution with glucuronic acid (Reis et al., 2002; 2003). The mixture of oligosaccharides obtained by acid hydrolysis was fractionated by size exclusion chromatography in order to enrich fractions in small groups of molecules for mass spectrometry analysis. By ESI-MS spectrum analysis, in positive mode, of oligosaccharides from a glucuronoxylan, the xylo-oligosaccharide structures identified corresponded to the series of neutral oligosaccharides of xylose, to the acidic oligosaccharides substituted by glucuronic acid residues, and also to the acidic oligosaccharides substituted with one 4-O-methyl-glucuronic acid residue. All these structures were confirmed by MS/MS spectra.

Mass spectrometry and tandem mass spectrometry have been successfully used for the identification of detailed structural features of coffee galactomannans and type II

arabinogalactans. Type II arabinogalactans from green coffee were isolated and, after a partial acid hydrolysis, were structurally characterized by mass spectrometry (Redgwell et al., 2002a; Nunes et al., 2008). Structural information of the arabinose side chains, as the O-5 position pentose residue linked, were obtained, as well as the presence of different oligosaccharides at O-3 position corresponding to side chains. Not many studies are found in the literature using mass spectrometry in galactomannans structural characterization, as well as arabinogalactans. Structural details of water soluble coffee polysaccharides have been determined, many of them with the help of structural studies by ESI-MS/MS of the oligosaccharides (Nunes et al., 2005; 2006a). The galactomannan structural features of green and roasted coffee infusions were elucidated by ESI-MS/MS analysis of the oligosaccharides obtained by specific enzymatic hydrolysis with the *Aspergillus niger endo*-β-mannanase (Nunes et al., 2005). The most abundant range of oligosaccharides was DP2 and DP3 (Fig. 1.5). Predominant $[M+Na]^+$ ions were observed at *m/z* 365 and 527, for di- and tri-hexoses (Hex_2 and Hex_3, respectively). The ion at *m/z* 407 corresponded to an acetylated disaccharide ($AcHex_2$) since a mass difference of 42 Da (acetyl group) between the ion at *m/z* 365 (Hex_2) and the ion at *m/z* 407 was observed. The ion at *m/z* 497, with a difference to *m/z* 365 of 132 Da (pentose residue), corresponded to the ion of the $PentHex_2$. These structural details, obtained by this MS ions analysis, were confirmed by MS/MS.

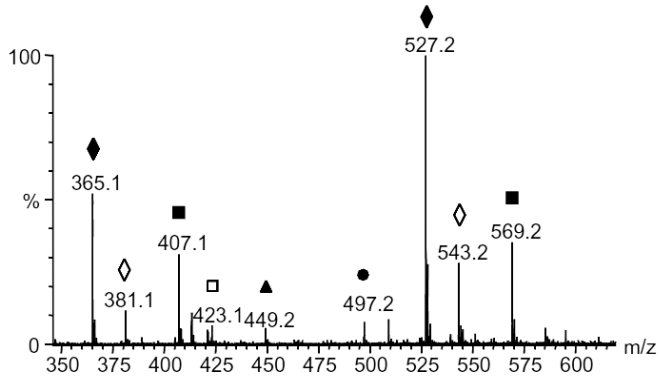


Fig. 1.5 *Positive ESI-MS of DP3 fraction obtained after enzymatic hydrolysis from roasted coffee (Nunes et al., 2005).*

As observed in figure 1.6, the MS/MS spectrum of $[PentHex_2+Na]^+$, at *m/z* 497 presented as the major fragment ion the ion at *m/z* 335, that corresponded to the loss of one hexose residue, attributed to $[PentHex+Na]^+$. It was also observed the fragment ion at *m/z*

365, attributed to $[Hex_2+Na]^+$, resulting from loss of 132 Da (pentose residue). The loss of 60, 90, and 120 Da from $[PentHex+Na]^+$, at *m/z* 335 (Fig. 1.6) showed the presence of the (1→6)-linkage of the pentose residue to the Hex.

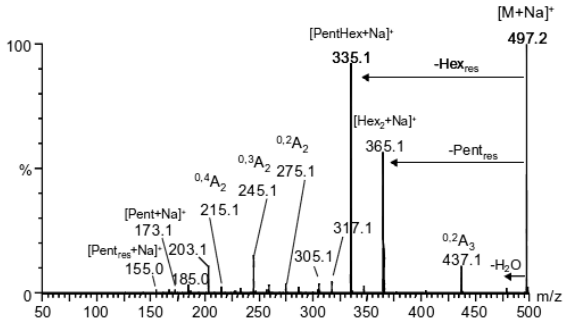


Fig. 1.6 *ESI-MS/MS spectra of $[M+Na]^+$ adducts of $PentHex_2$ (Nunes et al., 2005).*

Mass spectrometry and tandem mass spectrometry has been successfully used also for the roasting structural changes in coffee galactomannans. The roasting process induces structural changes in coffee galactomannans. These changes of galactomannans during the coffee roasting were detected by ESI-MS/MS (Nunes et al., 2006a). One of the most abundant modification were the 1,6-β-anhydromannose residues, a typical caramelization product, and a series of ions were present corresponded to the ions $[Hex_{1-4}AnHex+Na]^+$. Other two series identified were modified oligosaccharides by oxidation/decarboxylation, such as hexonic acid residues (HexA), present as $[Hex_{2-3}HexA+Na]^+$ and pentonic acid residues (PentA), present as $[Hex_2PentA+Na]^+$. A series of ions corresponding to oligosaccharides containing Amadori compounds of proline, valine, leucine/isoleucine, or 4-hydroxyproline and decarboxylated valine were also identified by ESI-MS and confirmed by ESI-MS/MS (Nunes et al., 2006a).

This versatility of mass spectrometry tools for analysis of oligosaccharides derived from polysaccharides were used in to exploit the structural details of galactomannans from different origins as well as the coffee galactomannans extracted under different conditions from coffee residue.

I.4 AIM OF THE WORK

As stated in the bibliography, coffee galactomannans have been shown to present *in vitro* immunostimulatory activity (Simões et al., 2009). These polysaccharides are present in high abundance in coffee residue, the by-product obtained after the preparation of the coffee brew. Due to the high coffee consumption, the coffee residue should be elected as a source of galactomannans with potential immunostimulatory activity. However, the use of coffee residue as a source of galactomannans has been hampered due to their insolubility in water upon extraction, as well as their low extractability from coffee residue (Simões et al., 2009). The acetylation of coffee residue polysaccharides presents a real possibility to render them soluble in water (Simões et al., 2009).

The chemically acetylated galactomannans obtained from coffee residue, the naturally acetylated galactomannans recovered from coffee brews, and the acemannans from *Aloe vera*, all presented *in vitro* immunostimulatory activity. However, the galactomannans from locust bean gum did not. In order to establish relationships between the structural features of these polysaccharides and their immunostimulatory activities, these polysaccharides were structurally analysed for sugars and linkage composition, selective *endo*-β-mannanase hydrolysis, and analysis of the resultant oligosaccharides by size-exclusion chromatography on Biogel P2 followed by tandem mass spectrometry of the low molecular weight compounds. The detailed characterization of coffee galactomannans are presented in chapter III.1, the chapter III.2 describes the locust bean gum structural features, and chapter III.3 presents the characterization of *Aloe vera* sample.

To improve the extractability of coffee by-product galactomannans, an experimental design was proposed based on the extractability of coffee galactomannans to the coffee brews and its increase with the increase of the degree of roast of the coffee bean (Nunes & Coimbra 2001; 2002a; 2002b). Also, because the extractability of the polysaccharides from the cell wall matrix is higher when the extraction is performed at higher temperatures, the increment of extractability was studied combining the roasting with hot alkali extractions. The coffee residue was roasted at 160 ºC and then submitted to alkali extractions with 4M NaOH solutions at 20, 60, and 120 ºC. The yields of the extractions were evaluated and the galactomannans recovered were characterized by sugars and linkage analysis, hydrolysed with a selective *endo*-β-mannanase, and the resultant products were fractionated by size-

exclusion chromatography on Biogel P2 followed by tandem mass spectrometry analysis. Also, the effect on cell walls structure of the successive alkali extractions was followed by scanning electron microscopy. These data and its discussion are presented in chapter 4.1. Chapter 4.2 is devoted to the thermal study of coffee residue extracted polysaccharides, namely the galactomannans and also the arabinogalactans. The thermograms obtained from 20 to 600 ºC at a heating rate of 10 ºC/min were compared with other polysaccharides, namely cellulose, locust bean gum, and gum Arabic was performed. The coffee polysaccharides were also submitted to an isothermal heating at different temperatures (160, 180, 200, 220, and 240 ºC) with long time of exposure, up to 3 h. The activation energies of thermal degradation were determined using both methods. The resultant products of thermal heating were analysed according to the sugars and linkage composition and also by electrospray mass spectrometry.

CHAPTER II

EXPERIMENTAL SECTION

II.1. SAMPLES ORIGIN

II.1.1. COFFEE, LOCUST BEAN GUM, AND *ALOE VERA* GALACTOMANNANS

To relate the immunostimulatory activity of galactomannans with their structural features, the samples available in our laboratory and previously studied by Simões et al. (2009) were used in sections III.1, III.2, and III.3:

The galactomannan-rich fraction from coffee infusion was provided by Prof. Fernando M. Nunes, from Universidade de Trás-os-Montes e Alto Douro, Vila Real, in Portugal. The fraction was obtained from the HMWM of a coffee infusion prepared from Brazilian Arabica coffee beans with a roast with 5% matter loss on a dry weight basis (DR 5%), as described by Nunes and Coimbra (2001; 2002a). This fraction was composed by mannose (89 mol%), galactose (7%), arabinose (2%), and glucose (1%). The total sugars were 76% and the amount of acetyl groups, on a molar basis in relation to the sugar residues, was 8% Nunes et al. (2005; 2006a).

Coffee residue was obtained from a commercial batch of Buondi coffee, a dark roasted Arabica coffee, roasted in Portugal and used to prepare espresso coffee. The galactomannan-rich fraction was obtained by 4 M NaOH and was solubilised by acetylation with acetic anhydride. This fraction was composed by mannose (69 mol%), galactose (23%), arabinose (6%), and glucose (2%). The total sugars were 55% and the amount of acetyl groups, on a molar basis in relation to the sugar residues, was 84% (Simões et al., 2009).

Two different sources of locust bean gum (LBG) galactomannans were used. LBG-FI was a commercial food industry sample from HG M200-INDAL-Faro, Portugal, provided by Prof. José A. Lopes da Silva, from the University of Aveiro. LBG-S was a commercial sample from Sigma Aldrich, used without any purification.

The *Aloe vera* galactomannan was obtained from a capsule of powder Aloe mucilaginous polysaccharides (AMP) extracted from *Aloe vera* plant, from Molo-Cure® (now called AMP Floracel®), USA, and containing 405.5 mg of powder.

II.1.2. COFFEE BY-PRODUCT

The coffee by-product used to study the polysaccharide extractability discussed, in section IV.1, and to study the thermal stability of the by-product, in section IV.2, was provided by the cafeteria of the Departament of Chemistry of the University of Aveiro. It was obtained from dark roasted Arabica coffee beans, from Buondi, Portugal, used to prepare espresso coffee.

II.1.3. POLYSACCHARIDES USED FOR THERMAL STUDIES

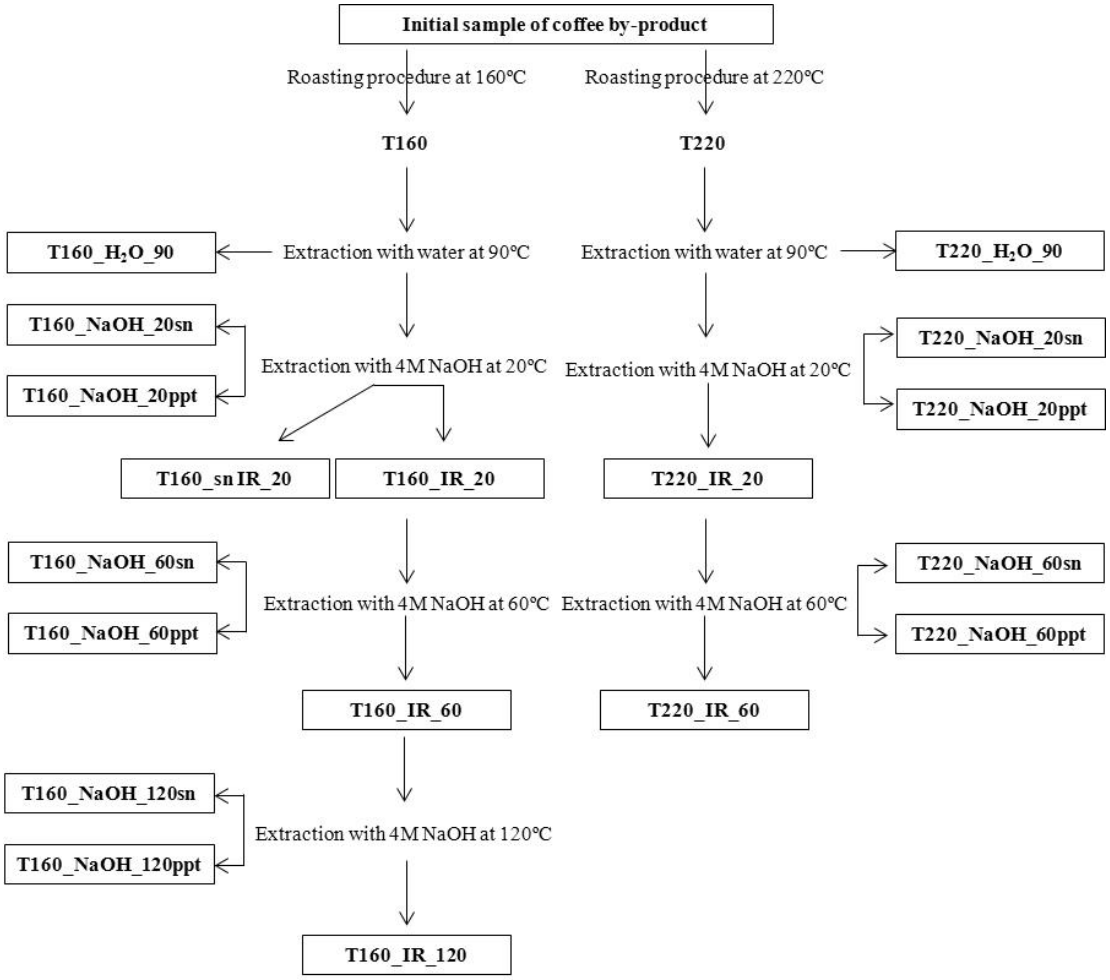
The thermal studies of the polysaccharides discussed in section IV.2 were performed using the galactomannan- (GM) and the arabinogalactan-rich (AG) extracts from coffee by-product (T160_NaOH_120ppt and T160_NaOH_120sn), as described in section II.2 and discussed in section IV.1. The LBG galactomannan used was the LBG-FI described in section II.1.1 and discussed in section III.2. The gum arabic was an arabinogalactan from acacia tree (Sigma) and cellulose was a microcrystalline powder obtained from J. T. Baker.

II.2. ROASTING OF COFFEE BY-PRODUCT

The coffee by-product was submitted to a roasting pre-treatment and then was sequentially extracted with hot water and alkali solutions (Scheme 2.1) in order to evaluate the possible increase of extraction yield of galactomannans. These results are discussed in section IV.1.

Samples of 500 g with 61% of moisture were submitted to a roasting procedure, one at 160 ºC (T160) and another at 220ºC (T220). These roasting procedures were done in a pre-heated oven (Binder) with an internal volume of 115 L, during 2 h for the assay at 160 ºC and during 1.5 h for the 220 ºC assays. After this period of time, at 220 ºC, carbonization of the coffee by-product occurred. Both coffee by-product samples submitted to a roasting pre-treatment were sequentially extracted with: a) distilled water at 90 ºC during 1 h, then b) with 4 M NaOH at room temperature (20 ºC), c) 4 M NaOH at 60

ºC and d) 4 M NaOH at 120ºC. All NaOH extractions were performed during 2 h. To prevent peeling reactions and alkaline oxidation of the polysaccharides, the NaOH extraction was carried out under an inert atmosphere (N_2) with O_2 free solutions containing 0.02 M $NaBH_4$. The NaOH solutions were prepared using distilled water previously boiled for 20 min and cooled under a nitrogen atmosphere. After each extraction step the mixture was filtered, concentrated under reduced pressure, and dialysed for 3 days, with several changes of distilled water. The final insoluble residue (IR) obtained were also dialysed for 3 days, with several changes of distilled water. The alkali extracts and IR extract were previously acidified to pH 5.0 with glacial acetic acid. After dialysis, the extracts were centrifuged and the precipitates (ppt), if present, were recovered separately from the supernatants (sn). The IR dialysed could be in same cases separated from another fraction, a supernatants (sn IR). All samples were frozen and freeze-dried.



Scheme 2.1. *Roasting procedure of coffee by-product and sequential extraction.*

II.3. ROASTING OF COFFEE GALACTOMANNANS AND ARABINOGALACTANS

In order to study the structural features that can be formed on coffee galactomannans and arabinogalactans, several roasting experiments were performed. The thermal behaviour of coffee polysaccharides were then compared with those of related polysaccharides, namely, LBG galactomannan, cellulose, and gum Arabic. The samples origin was described in section II.1.3 and results are discussed in section IV.2.

II.3.1. THERMOGRAVIMETRIC ANALYSIS

Thermogravimetric analysis (TGA) was carried out using a thermogravimetric analyzer (Shimadzu-50 automatic analyzer, Tokyo, Japan). Assays of 3-5 mg of coffee by-product sample, gum arabic, cellulose, LBG galactomannan, and coffee galactomannans were taken and warmed up from ambient to 600 °C at a heating rate of 10 °C/min in a dynamic (20 mL/min) air atmosphere. Thermogravimetric (TG) curves were analyzed using Shimadzu TASYS software.

II.3.2. THERMAL STABILITY OF COFFEE POLYSACCHARIDES

To study the thermal stability of coffee polysacharides, coffee galactomannans and arabinogalactans samples (15 ± 0.1 mg) were used in experiments under a controlled air flow of 20 mL/min, and a heating isothermically at 160, 180, 200, 220, and 240 ºC, during 3 h, using a thermogravimetric analyzer (Shimadzu-50 automatic analyzer, Tokyo, Japan).

In order to isolate the high from the low molecular weight material formed upon an extreme roasting, the galactomannan rich fraction from coffee by-product (GM, 0.6 g) was submitted to a roasting treatment at 240 ºC (GM_240_F) in an ashing furnace during 3 h. Under these experimental conditions, during the first 30 min the temperature was below 240 ºC. After 3 h at 240 ºC, the ashing furnace was turned off and the cooling process took place during 2 h. The 240 ºC roasted material was then dialyzed for 3 days, with several changes of distilled water. The high molecular weight material (GM_240_F_HMWM) and

the dialysate corresponding to the low molecular weight material (GM_240_F_ LMWM) were collected, frozen, and freeze-dried for further analysis.

II.3.3. ROAST OF MANNO-OLIGOSACCHARIDES

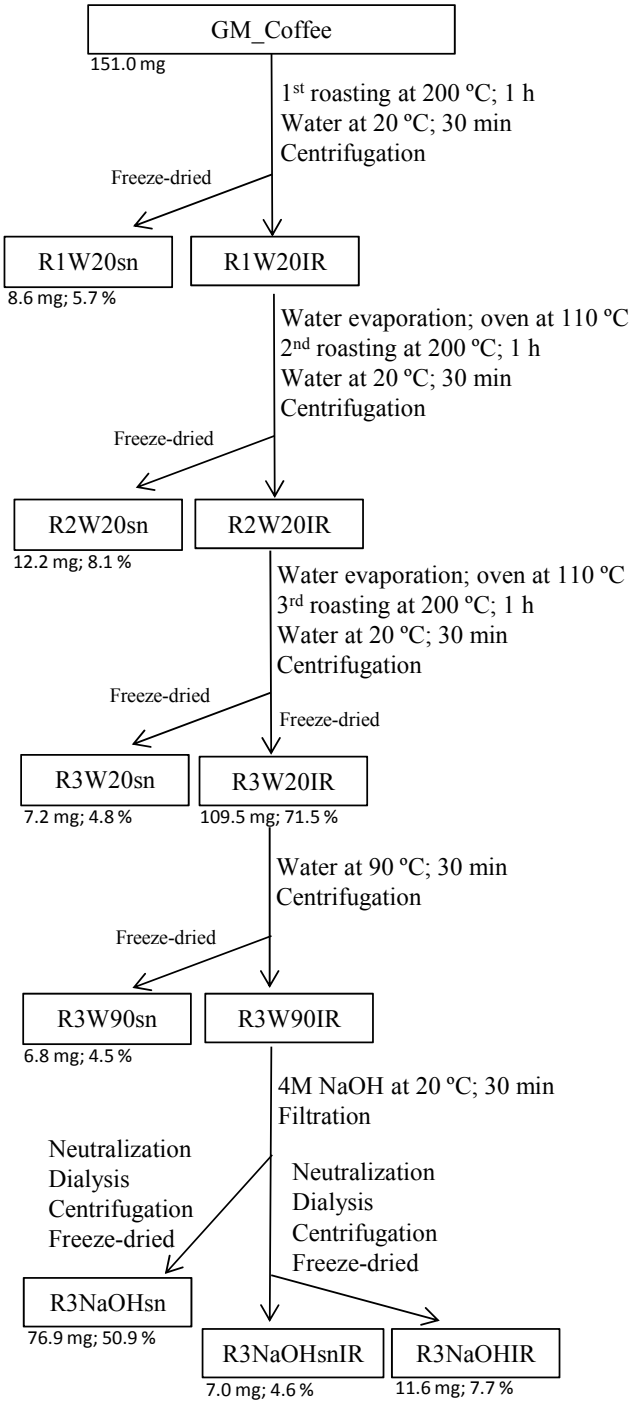
Oligosaccharide samples of β-(1→4)-D-mannotriose (Man_3) and β-(1→4)-D-mannotetraose (Man_4), from Megazyme, mimicking the mannan backbone, were also roasted for further analysis. The Man_3 and Man_4 samples (3-5 mg) were submitted to a 160 ºC and 200 ºC roasting procedure. These roastings were done during 2 h in a pre-heated oven (Binder) with an internal volume of 115 L.

II.4. SEQUENTIAL ROASTING PROCEDURES AND SOLUBILISATION OF GALACTOMANNANS

In order to try to convert the insoluble galactomannans recovered from coffee by-product into cold water soluble compounds, the water insoluble coffee galactomannan rich-fraction (1.0 g), described in section II.1.3, was submitted to a roasting treatment at 160 ºC, in a pre-heated oven (Binder) with an internal volume of 115 L during 1 h. After the roasting procedure, the material was suspended in 100 mL of water at room temperature (20 ºC) with stirring, during 1 h. The suspension was then centrifuged and the supernatant was separated from the insoluble residue and freeze-dried. The residue obtained was then dried at 110 ºC and then dispersed on a tray and submitted to another roasting treatment at 160 ºC during 1h. After the second roasting procedure at 160 ºC, the material was suspended in 100 mL of water at room temperature (20 ºC) with stirring, during 1h. This repetitive process of roasting at 160 ºC plus solubilization in water were done in a total of 8 times.

The water insoluble coffee galactomannan rich-fraction (151 mg) was also submitted to a roasting treatment at 200 ºC during 1 h. After the roasting procedure, the material was suspended in 50 mL of water at room temperature (20 ºC) with stirring, during 1h, as described for the 160 ºC. The suspension was then centrifuged and the supernatant (R1W20sn) was separated from the insoluble residue (R1W20IR) and freeze-dried. These assays were schematically registered in scheme 2.2., together with the

indication of the mass balance and respective yields of extraction. The residue obtained was then dried at 110 ºC and then dispersed on a tray and submitted to another roasting treatment at 200 ºC during 1h.



Scheme 2.2. *Coffee by-product galactomannans solubilisation by consecutive roasting procedure at 200 ºC and followed solubilisation in water and NaOH.*

After the second roasting procedure at 200 ºC, the material was suspended in 50 mL of water at room temperature (20 ºC) with stirring, during 1 h, allowing to obtain a new soluble fraction (R2W20sn) and a remaining insoluble residue (R2W20IR). This repetitive process of roasting at 200 ºC plus solubilization in water at 20 ºC was repeated a third time allowing to obtain the R3W20sn fraction and R3W20IR insoluble residue. The residue was then suspended in 100 mL of water at 90 ºC and was kept at 90 ºC under stirring during 30 min, allowing to obtain a soluble material (R3W90sn) that was separated from the residue (R3W90IR) by centrifugation. This residue was then dispersed in 100 mL of 4 M NaOH solution containing 0.02 M $NaBH_4$ at room temperature and left under stirring 30 min. The suspension was filtered and the alkali-soluble extract (R3NaOHsn) and the residue, after suspension in water, were acidified to pH 5.0 with glacial acetic acid dialyzed for 3 days with several changes of distilled water. After dialysis of the residue, the supernant solution that remained inside the dialysis membrane (R3NaOHsnIR) was separated by centrifugation from the insoluble material (R3NaOHIR) and both fractions were frozen and freeze dried (Coimbra et al., 1994). The yields of these procedures and the sugar linkage composition of the carbohydrates recovered are discussed in section IV.2.

II.5. ESTIMATION OF POLYSACCHARIDES MOLECULAR WEIGHT

In order to obtain an estimation of the molecular weight of the chemically acetylated coffee residue polysaccharides discussed in section III.1, a gel-filtration chromatography on Sephacryl S-300 HR (10– 400 kDa), Pharmacia, was performed on a 100 x 1.6 cm (XK 100/ 16, Pharmacia) column at a flow rate of 0.5 mL/min. The sample was suspended in 1 mL of 0.1 M potassium phosphate buffer pH 6.5 with 3 M urea. The same phosphate-urea buffer was used as eluent. Fractions (2 mL) were collected and were assayed for polysaccharides (phenol–sulfuric acid method) (Nunes & Coimbra 2001). To calibrate the column, standard dextrans of 25, 150, and 410 kDa (Sigma) were used. The column internal volume was determined by elution of glucose.

II.6. DETERMINATION OF THE DEGREE OF ACETYLATION

The degree of acetylation of the galactomannan-rich fractions discussed in sections III.1, III.2, and III.3 was determined by quantification of the acetic acid released by saponification of acetyl groups, acidification of solution, solid phase microextraction (SPME) and analysis by gas chromatography, in accordance to the method developed by Nunes e al. (2006b). The samples (2-3 mg) were dispersed in water (2.4 mL) in vials with 1 mL capacity and sonicated for 10 min in a water bath at room temperature. The saponification of the polysaccharides occurred by the addition of 0.8 mL of 2 M NaOH, with a reaction time of 1 h at 25 ºC. The reaction was finished by the addition of 0.8 mL of 2 M HCl, and the pH was adjusted to 2.0. The vials (10 mL) containing 4.0 mL of sample suspension (sample dispersed in 2.4 mL of water, 0.8 mL of 2 M NaOH and 0.8 mL of 2 M HCl) or standard solutions were thermostatized at 40 ºC in a water bath, with continuous stirring. After 15 min, the SPME fiber coated with 50/30 μm divinylbenzene/carboxen on polydimethylsiloxane (DVB/carboxen/PDM) was manually inserted through the Teflon septum into the headspace of the vial and exposed at 40 ºC during 30 min. The SPME coating fiber containing the headspace volatile compounds was introduced into the GC injection port at 250 ºC and kept for 10 min for the desorption. A Hewlett-Packard 5890 series II gas chromatograph (Hewlett-Packard, Wilmington, USA), equipped with a split/splitless injector and a flame ionization detector (FID) was used.

II.7. SPECIFIC HIDROLYSIS OF GALACTOMANNANS BY MANNANASE

Selective cleavage of the galactomannan backbone between adjacent β-(1→4)-linked mannose residues, allows to obtain mannan oligosaccharides that contain structural details that could be further analysed. This approach was used in sections III.1, III.2, III.3, and IV.1. Samples (15 mg) were hydrolyzed with 1 U of a pure *endo*-β-(1→4)-D-mannanase preparation (Megazyme, EC 3.2.1.78) during 48 h at 37 ºC with continuous stirring in a 100 mM Na-acetate buffer, pH 5.5, containing 0.02% sodium azide. The freeze-dried material was dissolved in water, and loaded on a XK 1.6/100 column containing Bio-Gel P-2 (Bio-Rad) previously equilibrated with water, and calibrated with DP4 (stachyose), DP2 (cellobiose) and monosaccharide (glucose), using a flow of 0.3

mL/min. Fractions (1 mL) were collected and assayed for sugars with evaporative light scattering detection. Fractions containing oligosaccharides were pooled and evaporated until all the eluent was removed. No lyophilization was performed since it has been shown that lyophilization promotes O-acetyl migration on galactomannans (Nunes et al., 2005).

II.8. STRUCTURAL ANALYSIS OF OLIGOSACCHARIDES BY MASS SPECTROMETRY

To study the structural features of polysaccharides, oligossacharides obtained after selective enzymatic hydrolysis treatment and further fractionated by size exclusion chromatography, were analysed by Electrospray ionization mass spectrometry (ESI-MS) and tandem-mass spectrometry (ESI-MS^n) in all discussion sections. To evaluate the presence of oligosaccharides with higher degree of polymerization than those observed by ESI-MS, a Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS) analysis was performed in section III.3 for *Aloe vera* structural features characterization.

II.8.1. ELECTROSPRAY IONIZATION MASS SPECTROMETRY (ESI-MS)

The fractions obtained after the Biogel P2 column after mannanase hidrolysis, described in section II.7, were disolved in water and further diluted in methanol/water/formic acid (50:49.9:0.1, v/v/v). Positive ion ESI-MS and MS/MS spectra were acquired using a LXQ linear ion trap mass spectrometer (Finningan). Typical ESI conditions were as follows: nitrogen sheath gas 30 psi, spray voltage 5 kV, heated capillary temperature 275 ºC, capillary voltage 1 V, and tube lens voltage 40 V. The flow rate was set to 8 μL/min and the voltage applied was 5 kV. Nitrogen was used as nebulizing and drying gas. Full scan mass spectra ranging from m/z 100 to 1500 were acquired in the positive mode. In the MS^2 experiments, collision energy varied between 15 and 25 of normalized collision. Data acquisition was carried out with Xcalibur data system.

II.8.2. MATRIX-ASSISTED LASER DESORPTION/IONIZATION MASS SPECTROMETRY (MALDI)

For MALDI analysis, 5 μL of sample dissolved in water were mixed with 20 μL of 2,5-dihydroxybenzoic acid (DHB) dissolved in a solvent mixture composed by acetonitrile:aqueous TFA (Trifluoroacetic acid) (0.1%, v/v) (70:30, v/v). From this mixture, 0.3 μL were deposited on top of a layer of crystals of 2-cloromercaptobenzothiazole (CMBT) formed by deposition of 0.5 μL of CMBT solution in tetrahydrofuran:methanol:water (1:1:1, v/v/v) on the MALDI plate (Pfenninger et al., 1999). MALDI mass spectra were acquired using a MALDI-TOF/TOF Applied Biosystems 4800 Proteomics Analyser (Applied Biosystems, Framingham, MA) instrument equipped with a nitrogen laser emitting at 337 nm and operating in a reflectron mode. Full scan mass spectra ranging from *m/z* 500 to 4000 were acquired in the positive mode.

II.9. SUGAR AND LINKAGE ANALYSES

Sugar and linkage analyses by methylation were performed to all samples used in the present PhD thesis. For LBG galactomannans, the oligosaccharides released upon *endo*-β-mannanase hydrolysis were directly reduced and permethylated with no hydrolysis, allowing their analysis by GC-MS (section III.2).

II.9.1. SUGAR ANALYSIS

Sugars were determined by gas chromatography (GC) as alditol acetates. The polysaccharides were treated with 72% (w/w) H_2SO_4 (10 mg/mL) during 3 h at room temperature with occasional stirring followed by hydrolysis for 2.5 h with 1 M or 2 M sulfuric acid at 100 ºC. Monosaccharides were reduced with $NaBH_4$ (15% in NH_3 3 M) during 1 h at 30 ºC and subsequent acetylated with acetic anhydride (3 mL) in the presence of 1-methylimidazole (450 μL) during 30 min at 30 ºC. Alditol acetate derivatives were separated with dichloromethane and analyzed by GC with a FID detector and equipped with a 30 m column DB-225 (J&W Scientific, Fol-som, CA, USA) with i.d. and film thickness of 0.25 mm and 0.15 μm, respectively. The oven temperature program used was: initial temperature 200 ºC, a rise in temperature at a rate of 40 ºC/min until 220 ºC, standing for 7 min, followed by a rate of 20 ◦C/min until 230 ºC and maintain this temperature 1 min. The injector and detector temperatures were, respectively, 220 and 230 ºC. The flow rate of the carrier gas (H_2) was set at 1.7 mL/min (Nunes & Coimbra, 2001). The hydrolysis of all samples was performed in duplicate.

II.9.2. LINKAGE ANALYSIS

Linkage analysis was carried out by methylation as described by Ciucanu and Kerek (1984). The sample was dissolved in 1 mL of anhydrous dimethylsulfoxide (DMSO), then powdered NaOH (40 mg) were added and samples were methylated with CH_3I (80 μL) during 20 min. Distilled water (2 mL) and dichloromethane (3 mL), were then added, and dichloromethane phase was washed three times by addition of distilled water (2 mL). The organic phase was evaporated to dryness and the material was remethylated using the same procedure (Nunes & Coimbra, 2001). The methylated material was hydrolyzed with TFA 2 M at 121 °C for 1 h, and then reduced and acetylated as previously described for neutral sugar analysis. The partially methylated alditol acetates (PMAA) were separated and analyzed by gas chromatography–mass spectrometry (GC–MS) on an Agilent Technologies 6890N Network. The GC was equipped with a DB-1 (J&W Scientific, Folsom, CA, USA) capillary column (30 m length, 0.25 mm of internal diameter and 0.15 μm of film thickness). The samples were injected in splitless mode (time

of splitless 5 min), with the injector operating at 220 ºC, and using the following temperature program: 45 ºC for 5 min with a linear increase of 10 ºC/min up 140 ºC, and standing for 5 min at this temperature, followed by linear increase of 0.5 ºC/min up to 170 ºC, and standing for 1 min at this temperature, followed by linear increase of 15 ºC/min up to 280 ºC, with further 5 min at 280 ºC. The helium carrier gas had a flow rate of 1.7 mL/min and a column head pressure of 2.8 psi. The GC was connected to an Agilent 5973 mass quadrupole selective detector operating with an electron impact mode at 70 eV and scanning the range *m/z* 40–500 in a 1 s cycle in a full scan mode acquisition.

II.9.3. PERMETHYLATED OLIGOSACCHARIDE ANALYSIS

The oligosaccharides from LBG released by the *endo*-β-(1→4)-D-mannanase were reduced with $NaBH_4$ (15% in NH_3 3 M) during 1 h at 30 ◦C. The excess of BH_4^- was destroyed by additions of several glacial acetic acid aliquots (10 μL). Methanol portions (3 mL) were added and the solutions were evaporated to remove the borate in the form of methyl borate esters. For methylation, the oligosaccharides were dissolved in 1 mL of dimethylsulfoxide (DMSO) and powdered NaOH (40 mg) was added. The solution was allowed to react during 30 min and then CH_3I (80 μL) was added, allowing to react for 1 h. The solution was diluted with 2 mL of distilled water and neutralized using 1 M HCl. The methylated OS were extracted with 3 mL of dichloromethane, being this phase washed by several additions of distilled water (2 mL). The organic phase was evaporated and the material was re-methylated by the same procedure to guarantee complete methylation. The reduced and permethylated OS were separated and analysed by gas chromatography–mass spectrometry (GC–MS) on an Agilent Technologies 6890N Network. The GC was equipped with a 400-1HT, dimethylpolysiloxan capillary column (25 m length, 0.22 mm of internal diameter, and 0.05 μm of film thickness). The samples were injected in splitless mode (time of splitless 2 min), with the injector operating at 220 ºC, and using the following temperature program: start at 100 ºC with a linear increase of 15 ºC/min up 350 ºC, and standing for 10 min at this temperature, The helium carrier gas had a flow rate of 0.2 mL/min and a column head pressure of 13.8 psi. The GC was connected to an Agilent 5973 mass quadrupole selective detector operating with an electron impact mode at 10 eV and scanning the range *m/z* 50-800 at 2 scans s^{-1}, in a full scan mode acquisition. Ion

extraction chromatograms were obtained from the full-scan acquisition mode using the ion at *m/z* 175, which is a diagnostic fragment ion of permethylated terminally-linked arabinose residue. Standards of mannose, β-(1→4)-D-mannobiose, α-(1→6)-D-galactosyl-β-(1→4)-D-mannobiose, β-(1→4)-D-mannotriose, α-(1→6)-D-galactosyl- β-(1→4)-D-mannotriose, β-(1→4)-D-mannotetraose, and α-(1→5)-L-arabinobiose were also reduced and permethylated and then analysed by the same procedure.

II.10. SCANNING ELECTRONIC MICROSCOPY (SEM) OF COFFEE RESIDUES

In order to follow the extraction of roasted coffe residue polysaccharides by the alkali solvents described in section II.2, SEM analysis were performed and discussed in section IV.1. Samples were fixed on steel supports and coated with 60% gold/ 40% palladium using a JEOL metalizer (FFC-1100, Tokyo, Japan) at 1100-1200 V, 5 mA for 10 min. A scanning electron microscope (Hitachi, S4100, Tokyo, Japan) at 25 kV was used.

CHAPTER III

STRUCTURE OF POLYSACCHARIDES WITH IMMUNOSTIMULATORY ACTIVITY

RESULTS AND DISCUSSION

III.1. STRUCTURAL FEATURES OF PARTIALLY ACETYLATED COFFEE GALACTOMANNANS PRESENTING IMMUNOSTIMULATORY ACTIVITY

HIGHLIGHTS:

► Immunostimulatory coffee infusion and coffee residue galactomannans have a comparable molecular weight and similar glycosidic-linkage composition. ► Galactomannans from coffee residue were preferentially acetylated in the side chain residues. ► Galactomannans from coffee infusions only had acetyl groups directly linked to the backbone residues. ► Coffee galactomannans from brew and residue present comparable immunostimulatory properties although different acetylation patterns.

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Structural features of partially acetylated coffee galactomannans presenting immunostimulatory activity

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Keywords: Coffee; Mannan; Acetylation of polysaccharides; Mass spectrometry; Electrospray; Oligosaccharides.

The galactomannans purified from coffee infusions present *in vitro* immunostimulatory activity on murine B- and T-lymphocytes. Galactomannans recovered from coffee residue by strong alkali solutions and rendered soluble in water by partial acetylation also showed similar immunostimulatory activity compared with coffee infusions. In this study, purified fractions of galactomannans with immunostimulatory activity obtained from coffee infusion and from coffee residue were compared according to their average molecular weight determined by size-exclusion chromatography on Sephacryl S300, glycosidic-linkage composition by methylation analysis, selective hydrolysis by an *endo*-β-(1→4)-D-mannanase, analysis of the resultant oligosaccharide profile by Bio-Gel P2 separation, and determination of the pattern of acetylation by electrospray tandem mass spectrometry (ESI-MS/MS).

III.1.1. Chemical characterisation of galactomannans

The results of methylation analysis of the galactomannans recovered from coffee residue and presenting immunostimulatory activity are shown in Table 3.1.1. These polysaccharides have a ratio of (1→4,6)-linked/Total Man of 0.078, which is indicative of a degree of branching (DB) of 8%, which is higher than the 4% observed for the galactomannans purified from coffee infusion. However, the ratio of total Man/terminally linked Man, diagnostic of the degree of polymerisation of the galactomannans, is comparable (23 for coffee residue and 20 for coffee infusion). These ratios are in the range of those found for roasted coffee infusion galactomannans (DB 4–5 and 17–24, respectively) (Nunes & Coimbra, 2001) and coffee residue galactomannans (DB 4%) (Redgwell et al., 2002b). Both samples contained a contamination of type II arabinogalactans, as infered by the presence of (1→3)- and (1→3,6)-linked galactose residues, as well as (1→5)-linked arabinose residues (3%). Also present were terminally-linked galactose and arabinose residues and (1→4)-linked glucose residues, which are known to be structural features of coffee galactomannans (Nunes et al., 2005). Nevertheless, the amount of type II arabinogalactans is higher in the coffee residue sample (24%) than in the coffee infusion used (3%).

Table 3.1.1 *Methylation analysis of the coffee infusion and residue galactomannans presenting immunostimulatory activity.*

Linkage	Coffee infusion[a] (mol%)		Coffee residue (mol %)	
T-Ara*f*	0.4		4.8	
5-Ara*f*	0.2		2.9	
Total	0.6 [b]	2[c]	7.6	6
T-Man*p*	4.6		3.0	
4-Man*p*	83.9		60.8	
4,6-Man*p*	3.3		5.4	
Total	91.8	89	69.1	69
T-Gal*p*	4.0		6.4	
6-Gal*p*	0.3		0.9	
3-Gal*p*	1.4		8.5	
3,6-Gal*p*	0.4		6.2	
Total	6.1	7	22.0	23
4-Glc*p*	1.8		1.2	
Total	1.8	2	1.2	2
4,6-Man*p*/Total Man*p*	0.036		0.078	
Total Man*p*/T-Man*p*	20		23	

[a]Values from Nunes et al., 2006a.

[b]Total molar percentage obtained by methylation analysis.

[c]Values are the molar percentage obtained by sugar analysis.

In order to obtain an estimation of the molecular weight of the polysaccharides present in coffee residue, a size-exclusion chromatography on Sephacryl-300HR was performed (Fig. 3.1.1). The average molecular weight was estimated as 109 kDa, which is a value similar to that obtained for the coffee infusion sample (140–90 kDa). The difference found for the molecular weight of coffee galactomannans determined by size-exclusion chromatography and endgroup analysis by methylation has been previously observed (Nunes & Coimbra, 2001), attributed to a possible degradation of the polysaccharides during methylation (Nunes & Coimbra, 2001). From these results, the primary glycan structure for the acetylated coffee residue galactomannans does not appear to differ significantly from the coffee infusion ones.

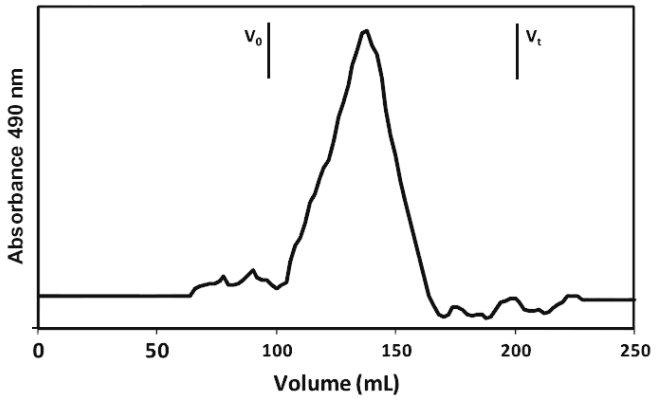


Fig. 3.1.1 *Size-exclusion chromatographic profile on Sephacryl-300 HR of galactomannans from coffee residue. V0, void volume and Vt, total volume.*

III.1.2. Selective hydrolysis of coffee galactomannans by an *endo*-β-(1→4)-D-mannanase

In order to evaluate and compare the acetylation pattern of these immunostimulatory galactomannans obtained from a roasted coffee infusion and from the residue, they were both selectively hydrolysed with an *endo*-β-(1→4)-D-mannanase. This selective degradation procedure allows cleaving the galactomannan backbone between adjacent β-(1→4)-linked mannose residues, allowing to obtain mannan oligosaccharides that contain structural details on the substituents of the mannan backbone (Dhawan & Kaur, 2007; Moreira & Filho, 2008). The oligosaccharides obtained by selective enzymatic hydrolysis of the mannan backbone were fractioned by size-exclusion chromatography, giving hydrolysis product profiles different for the two galactomannan samples (Fig. 3.1.2). The major oligosaccharides released from coffee infusion galactomannans were the disaccharides (DP2) and, in lower amount, the trisaccharides (DP3), although a small amount of monosaccharides, in the inclusion limit of the gel, and a small amount of oligosaccharides of higher degree of polymerisation, in the void volume of the gel (>2 kDa), were also present. Contrarily to what was observed for coffee infusion galactomannans hydrolysis products, the major oligosaccharides released for the coffee residue galactomannans were higher than DP3 and presented a heterogeneous degree of polymerization (Fig. 3.1.2). For these galactomannans, DP3 and DP2 oligosaccharides accounted for a small proportion of the total oligosaccharides released and monosaccharides were absent. Also, the carbohydrate material present in the void volume

of the gel was higher than that observed for the coffee infusion galactomannans.This high molecular weight material contains the contaminating Type II arabinogalactans present in this polysaccharide fraction. Also, because the galactomannans of coffee residue presented a high degree of acetylation, it is possible that the higher molecular weight carbohydrate fraction found in coffee residue galactomannans after enzymatic hydrolysis contains undegradable mannan portions for *endo*-β-(1→4)-D-mannanase, as the substitution pattern of mannans influences the range of oligosaccharides released after enzymatic hydrolysis (Daas et al., 2000).

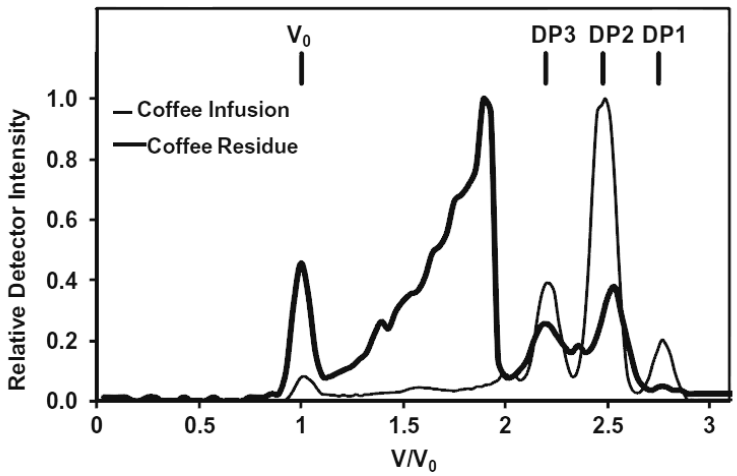


Fig. 3.1.2 *Size-exclusion chromatography on Bio-Gel P2 of the oligosaccharides obtained after enzymatic hydrolysis with an endo-b-D-mannanase of acetylated galactomannans from coffee infusion and coffee residue. V0- void volume, DP 2 and DP 3 correspond to the elution volume of DP 2 and DP 3 standard oligosaccharides, and DP 1 corresponds to the elution volume of monomers.*

III.1.3. ESI-MS analysis

To evaluate and compare the acetylation pattern of the naturally acetylated galactomannans of coffee infusion and the chemically acetylated galactomannans obtained from coffee residue (CR), the oligosaccharides obtained after mannanase treatment were analysed by mass spectrometry (MS) with electrospray ionization (ESI). The oligosaccharides identified as $[M + Na]^+$ ions in ESI-MS spectra from coffee infusion and coffee residue samples are shown in Table 3.1.2. ‘‘Hex” represents a hexose, as the *m/z* of Man, Gal or Glc is the same; ‘‘Pent” represents a pentose, probably Ara, as this was the

only pentose detected by sugar analysis and also identified in coffee infusion galactomannans, and ‘‘Ac” represents the acetyl groups.

Table 3.1.2*Oligosaccharide m/z ([M+Na]$^+$) ions observed by ESI-MS after enzymatic hydrolysis with an endo-β-(1→4)-D-mannanase of the mannans obtained from coffee infusion and coffee residue after chemical acetylation.*

	Coffee infusion		**Coffee residue**			
n	2	3	2	3	4	5
Hex_n	365	527	365	527	689	851
$PentHex_n$	497		497	659	821	
$AcHex_n$	407	569	407	569	731	893
$AcPentHex_n$		701	539	701	863	1025
Ac_2Hex_n	449	611		611	773	935
$Ac_2PentHex_n$		743	581	743	905	1067
Ac_3Hex_n					815	977
$Ac_3PentHex_n$			623	785	1079	1109

The number of oligosaccharide products observed for coffee residue galactomannan enzymatic hydrolysis was much higher than that observed for coffee infusion galactomannans, which is in accordance with the higher variability observed by the size-exclusion chromatographic profile. In coffee residue galactomannans it was identified di- to pentasaccharides constituted only by hexoses (Hex_{2-5}) whereas in coffee infusion galactomannans only di- and trisaccharides (Hex_{2-3}) were identified. As previously shown (Nunes et al., 2005), the mannanase hydrolysis of coffee infusion galactomannans gives origin to oligosaccharides containing pentoses. These oligosaccharides are also present in coffee residue galactomannans, allowing to infer the occurrence of galactomannans in coffee residue that present this structural feature previously observed in coffee infusion galactomannans (Nunes et al., 2005). From sugar analysis, this pentose should be arabinose.

Oligosaccharides containing acetyl groups were identified in both coffee galactomannan samples. It was possible to identify monoacetylated oligosaccharides and diacetylated oligosaccharides. Only for coffee residue galactomannans tri-acetylated hexose oligosaccharides have been observed (Table 3.1.2). This feature seems to be related with the higher degree of acetylation of the coffee residue galactomannan.

III.1.4. ESI-MS/MS analysis

In order to elucidate the structure of coffee oligosaccharides released from galactomannans, namely, the location of the acetyl groups on the original galactomannan polysaccharide backbone, they were submitted to a fragmentation under ESI-MS/MS conditions. ESI-MS/MS fragmentations are the result of glycosidic cleavages between two sugar residues and of cross-ring cleavages (cleavage of two bonds within the sugar ring) (Domon & Costello, 1988; Zaia, 2004). Analysing the ESI-MS/MS spectra of the $[M + Na]^+$ ions of acetylated oligosaccharides with the same molecular weight, and with the same sugars composition, but originated from different samples (coffee residue or coffee infusion galactomannan enzymatic hydrolysis products), it was possible to observe different fragmentations. The observed differences will allow to infer the presence of acetyl groups in different monosaccharide residues and thus to identify the acetylation pattern of each oligosaccharide (Nunes et al., 2006a; Reis et al., 2004).

To demonstrate this outcome, the ESI-MS/MS spectra of two pairs of isomeric oligosaccharides, $AcPentHex_3$, *m/z* 701, and $Ac_2PentHex_3$, *m/z* 743, obtained from the galactomannans from coffee infusion and coffee residue, are presented in Figs. 3.1.3 and 3.1.4, respectively. Comparing the MS/MS spectra of these pairs of isomers it is possible to see that the MS/MS spectra are distinct, revealing differences in the fragmentation pattern of these ions with the same *m/z* value, indicating different structures for the oligosaccharides obtained from the chemically and naturally acetylated galactomannans.

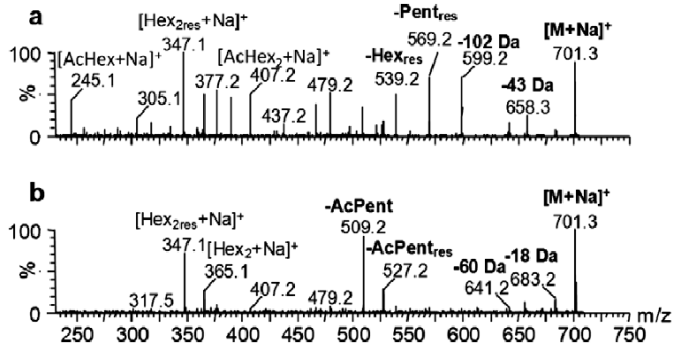


Fig. 3.1.3 *ESI-MS/MS spectra of the $[M + Na]^+$ adducts of $AcPentHex_3$ (m/z 701) obtained respectively from (a) coffee infusion and (b) coffee residue.*

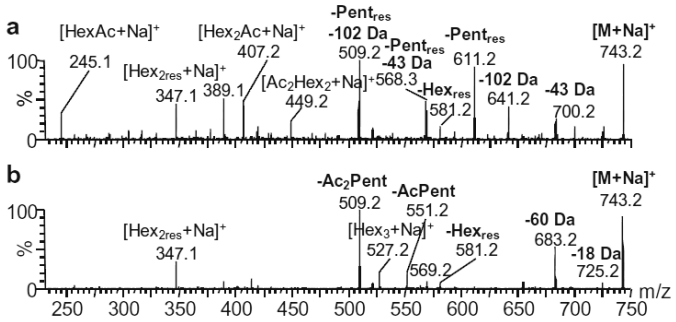
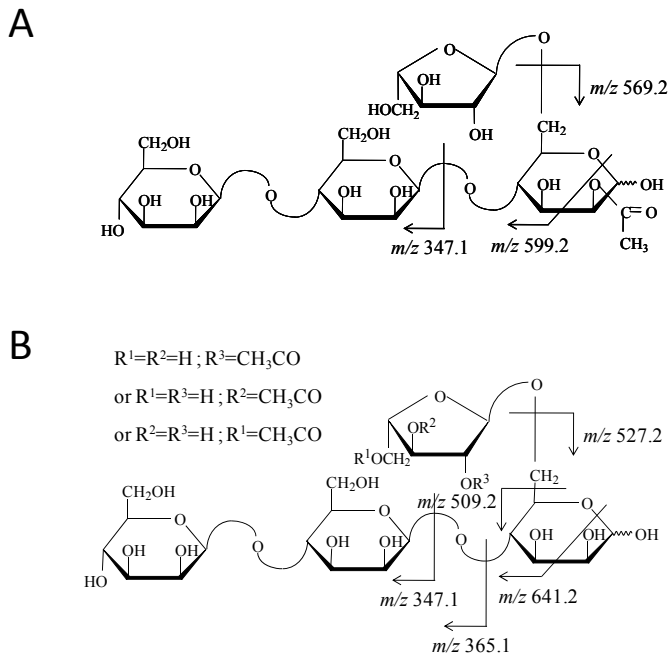


Fig. 3.1.4 *ESI-MS/MS spectra of the $[M+Na]^+$ adducts of $Ac_2PentHex_3$ (m/z 743) obtained respectively from (a) coffee infusion and (b) coffee residue.*

III.1.4.1. ESI-MS/MS analysis of monoacetylated tetrasaccharides containing a pentose

The ESI-MS/MS spectrum of $[AcPentHex_3 + Na]^+$ (*m/z* 701) ions from coffee infusion sample showed the presence of ions at *m/z* 569, 407, and 245, identified as $[AcHex_3 + Na]^+$, $[AcHex_2 + Na]^+$, and $[AcHex + Na]^+$, resultant of loss of one $Pent_{res}$ (132 Da), one $Pent_{res}$ plus Hex_{res} (294 Da), and one $Pent_{res}$ plus two Hex_{res} (294 Da), respectively (Fig. 3.1.3a and Scheme 3.1.1). Also observed with a high abundance was the ion at m/z 347, identified as $[Hex- Hex_{res} + Na]^+$, resultant from loss of AcPentHex (354 Da). These results show that this oligosaccharide is acetylated in one of the hexoses, excluding the non-reducing mannose. The presence of one acetyl group is also noticed by the ions at *m/z* 658 and 479, resultant of loss of $-CH_3CO^{\cdot}$ (43 Da) and AcHex (222 Da), respectively (Fig. 3.1.3a). The loss of AcHex (222 Da) suggests the presence of an isomeric structure where the Pent is linked to the inner mannose residue and the acetyl group is linked to the mannose at the reducing end. The occurrence of the ion at *m/z* 599, resultant from loss of 102 Da, attributed to a $^{0,2}A_3$ cross ring fragment containing the acetyl group at O-2 position (Nunes et al., 2005), shows that the acetyl group is linked to the O-2 of the hexose residue of the reducing end terminal (Fig. 3.1.3a and Scheme 3.1.1).



Scheme 3.1.1. *Fragmentation pattern observed in the ESI-MS/MS spectra of the $[M+Na]^+$ adducts of AcPentHex$_3$ (m/z 701) obtained respectively from (A) coffee infusions and (B) coffee residue.*

The ESI-MS/MS spectrum of the $[AcPentHex_3 + Na]^+$ (*m/z* 701) ion from coffee residue sample (CR, Fig. 3.1.3b) shows a different fragmentation pattern when compared with the one reported above for the isomeric oligosaccharide from coffee infusion. In this MS/ MS spectrum, the major ions are observed at *m/z* 509 $[Hex_{3res} + Na]^+$ and 527 $[Hex_3 + Na]^+$, resultant from the loss of an acetylated pentose, AcPent (192 Da), and loss of an acetylated pentose residue, $AcPent_{res}$ (-174 Da) (Scheme 3.1.1B). Also, the ion resultant of the loss of $Pent_{res}$ (132 Da) at *m/z* 569, correspondent to $[AcHex_3 + Na]^+$, which is the major product ion of the MS/MS spectra of coffee infusion oligosaccharide, is absent in this spectrum of coffee residue. In addition, the ion at *m/z* 245, correspondent to $[AcHex + Na]^+$, is also absent. These results allow to conclude that the chemically acetylated galactomannan from coffee residue is acetylated in the pentose residue, a structural feature not observed for the naturally acetylated coffee infusion galactomannan. The information obtained from the MS/MS spectra does not allow to exactly locating the esterified position on the pentose residue. The ions $[HexHex_{res} + Na]^+$, at *m/z* 347, and $[Hex_2 + Na]^+$, at *m/z* 365, formed by the combined loss of AcPent plus Hex_{res}, and Ac-$Pent_{res}$ plus Hex_{res}, respectively, are also observed with high abundance. Ions due to loss of $Pent_{res}$ are absent in this spectrum. The ion at *m/z* 407, correspondent to $[AcHex_2 + Na]^+$ was observed, but

in very low relative abundance, indicating that acetylation also occurs at the hexose residues, although in a low abundance. The absence of ions resultant of loss of 102 Da ($^{0,2}A_3$ cross ring fragment containing the acetyl group at O-2 position) and of the ion at *m/z* 658, resultant of a loss of 43 Da ($-CH_3CO^{\cdot}$) show that the terminal mannose residue of this oligosaccharide is not acetylated. These features are also observed for the other oligosaccharides with higher degrees of polymerisation and related structure ($AcHex_nPent$, results not shown).

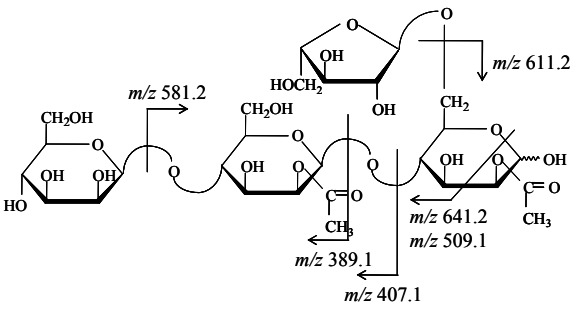
III.1.4.2. ESI-MS/MS analysis of diacetylated tetrasaccharides containing a pentose

The ESI-MS/MS spectrum of the isomeric diacetylated oligosaccharide $[Ac_2PentHex_3 + Na]^+$ (*m/z* 743) ions obtained from coffee infusion and coffee residue galactomannans are shown in figure 3.1.4. The oligosaccharides obtained from coffee infusion (Fig. 3.1.4a) presented the fragmentations due to the loss of $Pent_{res}$ (ion at *m/z* 611), loss of Hex_{res} (ion at *m/z* 581), and combined loss of $Pent_{res}$ plus Hex_{res} (ion at *m/z* 449), thus indicating that the pentose residue is not acetylated neither the hexose from the non-reducing end. Abundant ions due to loss of 102 and 43 Da from the $[M + Na]^+$ (ions at *m/z* 641 and 700) and from $[(M\text{-}Pent_{res} + Na)]^+$ (ions at *m/z* 509 and 568), resultant from cross ring and acetyl group cleavages, and the ion at *m/z* 245, attributed to $[AcHex + Na]^+$, were also observed, indicating that one acetyl group is at the C-2 of the reducing end mannose residue (Fig. 3.1.4a., Scheme 3.1.2A). All these fragmentations confirmed the presence of the acetyl groups linked to galactomannan backbone.

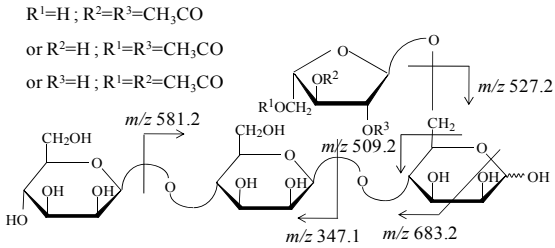
The ESI-MS/MS spectrum of the $[Ac_2PentHex_3 + Na]^+$ ion, at *m/z* 743, obtained from coffee residue (Fig. 3.1.4b, Scheme 3.1.2B) shows fragments due to the loss of Ac_2Pent and Ac_2Pent_{res}, with formation of the ions at *m/z* 509, correspondent to $[Hex_{3res} + Na]^+$, and 527, correspondent to $[Hex_3 + Na]^+$, respectively, as resumed in Scheme 4B1. These fragmentations indicate that both acetyl groups are linked to the pentose residue that is linked to the mannan backbone. From the MS/MS data no evidence of the exact location of the acetyl groups can be achieved. The combined loss of Ac_2Pent plus Hex_{res} (ion at *m/z* 347) is also observed (Scheme 3.1.2B1). Another fragmentation pathway correspondent to

the loss of AcPent is observed by the ion at *m/z* 551, with formation of the ion $[AcHex_2\text{-}Hex_{res} + Na]^+$ and loss of $AcPent_{res}$, by formation of the ion at *m/z* 569, correspondent to $[AcHex_3 + Na]^+$. These ions show the presence of an isomeric oligosaccharide structure bearing one acetyl group linked to the pentose residue and another linked to a hexose residue (Scheme 3.1.2B2). As observed previously for the tetrasaccharides, the product ion spectra of coffee residue oligosaccharides do not show loss of $Pent_{res}$ (-132 Da), loss of 102 Da neither loss of 43 Da. The absence of the cross ring fragmentation ($^{0,2}A_3$ cross ring fragment containing the acetyl group at O-2 position) and the absence of the ion formed by the loss of 43 Da ($-CH_3COO^-$) show that the terminal mannose residue of this oligosaccharide is not acetylated. These fragmentation pathways were also observed for the other ions of the series $Ac_2PentHex_n$. Thus, it is possible to conclude that the acetylation in these oligosaccharides from chemically acetylated mannans occurred also preferentially in the pentose ramification.

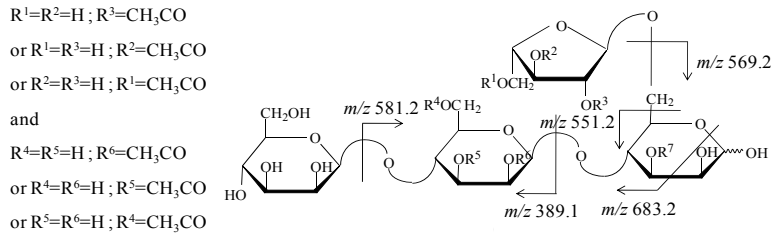
A



B1



B2



Scheme 3.1.2. *Fragmentation pattern observed in the ESI-M/MS spectra of the $[M+Na]^+$ adducts of $Ac_2PentHex_3$ (m/z 743) obtained respectively from coffee (A) infusion and (B1 and B2) residue.*

III.1.5. Concluding remarks

The chemically acetylated galactomannans isolated from coffee residue have a degree of branching and a molecular weight similar to those found for coffee infusion galactomannans. Nevertheless, the acetylation pattern and the degree of acetylation of these galactomannans were significantly different from the natural acetylation pattern and degree of acetylation occurring in coffee infusion galactomannnans. Whereas the acetylation of coffee infusion galactomannans occurs in the mannose residues of the backbone, preferentially at the O-2 position, the acetylation of the chemically acetylated coffee residue galactomannans occurs preferentially at the side chains, here demonstrated for the pentose residues present as substituents of the coffee mannan backbone. Nevertheless, the galactose residues present in coffee galactomannans as side chains should also be acetylated. However, since they have the same molecular weight as the mannose residues of the backbone, it is not possible to differentiate their presence from the acetylated mannose residues. These results show that these polysaccharides that present comparable immunostimulatory properties have different acetylation patterns, suggesting that further structure-activity studies need to be performed in order to understand their action.

RESULTS AND DISCUSSION

III.2. DEMONSTRATION OF THE PRESENCE OF ACETYLATION AND ARABINOSE BRANCHING AS STRUCTURAL FEATURES OF LOCUST BEAN GUM

HIGHLIGHTS:

► LBG galactomannans were submitted to a mannanase hydrolysis and the resultant oligosaccharides were analysed by ESI-MS/MS. ► Acetylated hexoses and/or substituted with a pentose were found. ► These structures were confirmed by GC–MS of reduced and permethylated oligosaccharides. ► Glycosidic-linkage analysis shows the occurrence of Ara*f* linked at the O-6 of (1 → 4)-Man*p* residues.

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Demonstration of the presence of acetylation and arabinose branching as structural features of locust bean gum galactomannans

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In the previous chapter the structural features of the galactomannans purified from coffee infusions and coffee residue, both presenting *in vitro* immunostimulatory activity on murine B- and T-lymphocytes, were determined. These polysaccharides, although presenting comparable immunostimulatory properties, had different acetylation patterns. However, the galactomannans from locust bean gum (LBG), although containing also single Gal*p* residues as side chains in a β-(1→4)-Man*p* backbone, did not present immunostimulatory activity (Simões et al., 2009).

LBG is a white to yellowish powder obtained by crushing the endosperm of the seeds of the fruit pod of carob tree (*Ceratonia siliqua* L.), a large leguminous evergreen found in Mediterranean regions (Dea & Morrison, 1975). The LBG is widely used in food industry as viscosifier, stabilizer, and gelling agent due to its low cost and wide range of functional properties (Fox, 1997). It has found increasing applications in the pharmaceutical industry (Mu et al., 2003; Sujja-areevath et al., 1998), as well as in textiles, paper, and cosmetics, among other industries. Its mixture with other polymers, namely carrageenan, agar, or xanthan, form more elastic and stronger gels (Andrade et al., 2000; Bresolin et al., 1999; Hernandez et al., 2001) allowing to structure the systems, improving

the application scale and the consumer acceptability of the products. The galactomannans are the main component of LBG (80–91%), with protein accounting for 5–6%, 1–4% cellulose, and 1% of ashes (Glicksman, 1969). Although all these applicatons, the detailed structure of these galactomannans still remains uncertain, namely concerning to the presence of sugars other than mannose and galactose that are always detected, namely, arabinose and glucose, although in small amounts.

In order to better understand what are the structural features of LBG galactomanans that may prevent their immunostimulatory activity when compared with coffee galactomannans, the galactomannans from LBG obtained from two different commercial sources were submitted to a selective degradation by *Aspergillus niger endo*-β-D-mannanase and the oligosaccharides (OS) obtained were fractioned by size exclusion chromatography using a Biogel-P2 (100–1800 Da). The oligosaccharides obtained were analysed by Electrospray Tandem Mass Spectrometry (ESI-MS^n) and confirmed by GC–MS of reduced and permethylated oligosaccharides.

III.2.1. Chemical characterization of LBG extracts

LBG-FI and LBG-S sugars composition is presented in Table 3.2.1 Mannose (77 and 73%, respectively) is the main sugar residue, followed by galactose (22 and 23%), glucose (1 and 3%), and arabinose (traces and 2%). This allows to calculate a Man/Gal ratio of 3.5 for LBG-FI and 3.2 for LBG-S. These values are in agreement with the values between 3 and 4 reported in the literature (Dakia et al., 2008; Kok, 2007; Lazaridou et al., 2000; Sittikijyothin et al., 2005).

Table 3.2.1 *Sugar analysis of LBG samples.*

	Sugar Composition (%mol)				% Total Sugars	Man/Gal
	Ara	Man	Gal	Glc		
LBG- FI	t	77	22	1	91.4	3.5
LBG- S	2	73	23	3	74.0	3.2

t-traces

The results of methylation analysis of the galactomannans from LBG samples are shown in Table 3.2.2. LBG-FI and LBG-S samples contained 59.2 and 54.6%, respectively, of (1→4)-linked mannose residues, 19.3 and 18.4% of (1→4,6)-linked, and 1.3 and 10% of terminally linked mannose residues. The terminally linked galactose residues were 19.3 and 16.2%. The sugars composition (Table 3.2.1) and methylation analysis of the LGB polysaccharides (Table 3.2.2) confirm the presence of galactomannans in both LBG samples. Both polysaccharides have similar ratio of total Man/(1→4,6)-linked Man, of 4.1 for LBG-FI and 4.0 for LBG-S, which shows an average of one branched residue in each four Man residues. The ratio of total Man/terminally-linked Man, which allows estimating the average degree of polymerisation of the galactomannans, is 63 for LBG-FI and 73 for LBG-S. In both samples, it was found that all arabinose residues were terminally-linked, presenting LBG-S a higher amount than LBG-FI, in agreement with sugars analysis.

Table 3.2.2 *Glycosidic-linkage composition of LBG samples.*

	LBG- FI	LBG- S
Linkage	Area (%)	
T-Ara*f*	0.1	1.4
T-Man*p*	1.3	1.0
4-Man*p*	59.2	54.6
4,6-Man*p*	19.5	18.4
T-Gal*p*	19.3	16.2
T-Glc*p*	0.0	1.3
4-Glc*p*	0.6	7.1
Total Man*p*/T-Man*p*	63	73
Total Man*p*/4,6-Man*p*	4.1	4.0

According to Table 3.2.1, the LBG-FI sample is richer in sugars (91.4% of total sugar) than LBG-S (74.0%), confirming the purification step reported to be performed on LBG-FI. However, the different purities of the samples used do not interfere in the specific enzymatic hydrolysis with the *endo*-β-(1→4)-D-mannanase done. The *endo*-β-(1→4)-D-mannanase, cleaving the β -(1→4)-Man linkages in the mannan backbone, allows the resultant oligosaccharides to be obtained from the galactomannans under study. If the arabinose found in sugar analysis is a component of the galactomannans it should be present in the low molecular weight material resultant of the enzymatic hydrolysis, as reported to occur in galactomannans of coffee (Nunes et al., 2005; Chapter III.1). The occurrence of arabinose in galactomannan-rich extracts has been reported in a crude locust bean gum (Kok, 2007), as well as in extracts of galactomannans from other sources, such as *G. triacanthos* (Navarro, Cerezo, & Stortz, 2002) and *A. pavonina* and *C. pulcherrima* (Cerqueira et al., 2009). It is still uncertain if this is a structural feature of these polysaccharides or if this is an impurity.

III.2.2. Selective hydrolysis of LBG galactomannans by an *endo*-β-(1→4)-D-mannanase

The hydrolysis with the *endo*-β-(1→4)-D-mannanase allows a selective cleavage of the galactomannan backbone between adjacent β-(1→4)-linked mannose residues, allowing to obtain mannan oligosaccharides that contain structural details on the substituents of the mannan backbone (Dhawan & Kaur, 2007; Moreira & Filho, 2008). According to the known enzymatic mechanism of Aspergillus niger endo-β-mannanase, the hydrolysis of the β -(1→4) linked mannan backbone is hindered by the presence of substituted residues, resulting in linear and branched di- and trisaccharides (DP2 and DP3) that were separated by size-exclusion chromatography on Biogel-P2 (Fig. 3.2.1). Oligosaccharides with a higher degree of polymerisation were also present. These compounds resistant to endo- β -mannanase hydrolysis are expected to be made up of higher branched structures and/or by the presence of β-(1→4)-Glc, as reported to occur in galactomannans of other sources, such as coffee (Nunes et al., 2005). The presence of (1→4)-linked Glc in both samples (Table 3.2.2), especially in the purified one, suggests that LBG contains also Glc interspersed in the mannan backbone.

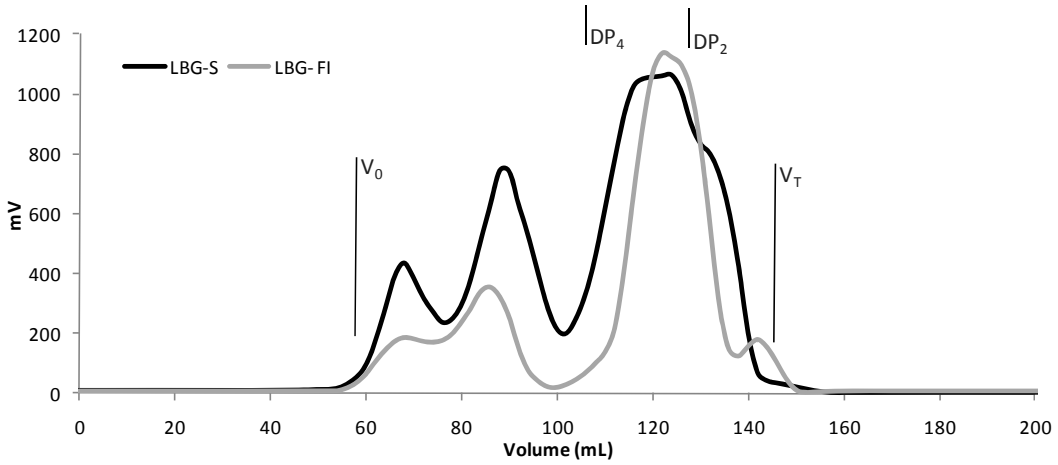

Fig. 3.2.1 *Size-exclusion chromatography on Bio-Gel P2 of the OS obtained after enzymatic hydrolysis with an endo-β-(1→4)-D-mannanase of galactomannans from LBG-S and LBG-FI. V0 – void volume; VT – elution volume of monomers.*

III.2.3. Characterization of LBG galactomannans by electrospray mass spectrometry

To study the structural features of LBG galactomannans, the oligosaccharides (OS) obtained after selective hydrolysis with an endo-β-mannanase treatment and further fractionated by size exclusion chromatography were analysed by mass spectrometry (MS) with electrospray ionization (ESI). The OS identified are summarized in Table 3.2.3 with indication of the *m/z* value of the $[M+Na]^+$ ions. "Hex" represents a hexose, as Man, Gal, and Glc cannot be distinguished because they have the same molecular weight; "Pent" represents a pentose, probably Ara, as this was the only pentose detected by sugar and methylation analysis, and "Ac" represents the acetyl groups.

By ESI-MS, based on the correspondent molecular weight, it were identified, in both samples of LBG galactomannans, $[M+Na]^+$ ions of OS composed by hexose residues (Hex_n, n = [3–6]), the ions present in higher relative abundance in the mass spectra (Table 3.2.3). It was also observed, although with a small relative abundance, $[M+Na]^+$ ions corresponding to OS with hexoses and a pentose residue ($PentHex_n$, n = [3–6]), and also hexose OS with an acetyl group ($AcHex_n$, n = [3–6]). Furthermore, OS with a pentose and acetyl groups ($AcPentHex_n$, n = [3–6]) were also found in the ESIMS spectra of both samples. Galactomannans bearing a pentose residue and/or acetyl groups have previously been observed in coffee (Nunes et al., 2005; Chapter III.1).

Table 3.2.3 *Oligosaccharide $[M+Na]^+$ ions observed in the ESI-MS spectra of the several fractions obtained after enzymatic hydrolysis with an endo-β-(1→4)-d-mannanase of the mannans obtained from LBG-FI and LBG-S.*

n	3 *(m/z)*	4 *(m/z)*	5 *(m/z)*	6 *(m/z)*
LBG (FI and S)				
Hex_n	527	689	851	1013
$AcHex_n$	569	731	893	1055
$PentHex_n$	659	821	983	1145
$PentAcHex_n$	701	863	1025	1187

III.2.4. ESI-MS/MS analysis

In order to confirm the presence of pentose residues and acetyl groups as structural features of LBG, the OS were also studied by ESI-MS/MS. With this method, it is possible to observe product ions formed by glycosidic cleavages between two sugar residues that allow confirmation of the sugar composition and also product ions formed by cross-ring cleavages that can give information about the type of linkages (cleavage of two bonds within the sugar ring) (Domon & Costello, 1988; Zaia, 2004). The analysis of the $ESIMS^n$ spectra allowed the validation of the structures of OS observed by ESI-MS and proposed in Table 3.2.3. To demonstrate this outcome, the product ion spectra ($ESI-MS^n$) obtained for the ions $[M+Na]^+$ of $AcHex_6$ (*m/z* 1055), $PentHex_6$ (*m/z* 1145), and $PentAcHex_6$ (*m/z* 1187) will be described in detail. Data obtained from the ESI-MS/MS spectra from the other OS identified are summarized in Table 3.2.4.

Table 3.2.4 *Product ions and their corresponding neutral losses identified in the ESI-MS/MS spectra of the LBG OS of the series $Pent_mAc_nHex_6$ and ESI-MS^3 of the LBG OS $Pent_mAc_nHex_5$, [m,n] = [0,1]; [1,0]; [1,1] and Ac_nHex_6, n = 0–1, with the indication of the m/z values.*

		MS^2 $AcHex_6$	MS^3 $AcHex_5$	MS^2 $PentHex_6$	MS^3 $PentHex_5$	MS^3 Hex_6	MS^2 $PentAcHex_6$	MS^3 $PentAcHex_5$	MS^3 $AcHex_6$
		m/z							
m/z	**Fragment ions**	**1055**	**893**	**1145**	**983**	**1013**	**1187**	**1025**	**1055**
1055	$AcHex_6$						$-Pent_{res}$		
1013	Hex_6			$-Pent_{res}$					
1025	$PentAcHex_5$						$-Hex_{res}$		
1007	$PentAcHex_{5res}$						-Hex		
983	$PentHex_5$			$-Hex_{res}$					
965	$PentHex_{5res}$			-Hex					
893	$AcHex_5$	$-Hex_{res}$							$-Hex_{res}$
875	$AcHex_{5res}$	-Hex							-Hex
851	Hex_5				$-Pent_{res}$	$-Hex_{res}$			
833	Hex_{5res}	-AcHex				-Hex			-AcHex
863	$PentAcHex_4$							$-Hex_{res}$	
845	$PentAcHex_{4res}$							-Hex	
821	$PentHex_4$			$-Hex_{2res}$	$-Hex_{res}$		$-AcHex_{2res}$		
803	$PentHex_{4res}$			$-Hex_2$	-Hex				
731	$AcHex_4$	$-Hex_{2res}$	$-Hex_{res}$						
713	$AcHex_{4\ res}$	$-Hex_2$	-Hex					$-PentHex_{res}$	
689	Hex_4	$-AcHex_{2res}$		$-PentHex_{2res}$	$-PentHex_{res}$	$-Hex_{2res}$	$-PentAcHex_{2res}$		$-AcHex_{2res}$
671	Hex_{4res}	$-AcHex_2$	-AcHex	$-PentHex_2$	-PentHex	$-Hex_2$			$-AcHex_2$
683	$PentAcHex_3$							$-Hex_{2res}$	
659	$PentHex_3$			$-Hex_{3res}$	$-Hex_{2res}$				
641	$PentHex_{3res}$			$-Hex_3$	$-Hex_2$				
569	$AcHex_3$	$-Hex_{3res}$	$-Hex_{2res}$						
551	$AcHex_{3res}$	$-Hex_3$	$-Hex_3$						
527	Hex_3	$-AcHex_{3res}$			$-PentHex_{2res}$	$-Hex_{3res}$			
509	Hex_{3res}	$-AcHex_3$	$-AcHex_2$	$-PentHex_3$	$-PentHex_2$	$-Hex_3$			
407	$AcHex_2$		$-Hex_{3res}$						
389	$AcHex_{2res}$		$-Hex_3$						
347	Hex_{2res}		$-AcHex_3$	$-PentHex_4$	$-PentHex_3$	$-Hex_4$			

III.2.4.1. ESI-MS^n of $AcHex_6$

Tandem mass spectra of Hex_n have previously been described in detail in previous studies (Chapter III.1), showing successive losses of Hex_{res} (-162 Da). The ESI-MS/MS spectrum of the ion $[AcHex_6+Na]^+$, at *m/z* 1055, observed for LBG samples, is shown in figure 3.2.2a. In this spectrum it is possible to observe the product ions formed by loss of one (-162 Da), two (-2*162 Da) and three (-3*162 Da) hexose residues with formation of

sodium adducts of acetylated OS, respectively, $[AcHex_5+Na]^+$ at *m/z* 893, $[AcHex_4+Na]^+$ at *m/z* 731, and $[AcHex_3+Na]^+$ at *m/z* 569, confirming that this is an OS composed by hexose units. It is possible to see the product ions at *m/z* 833, identified as $[Hex_5res+Na]^+$, formed by loss of one AcHex (loss of 222 Da). This identification confirms the occurrence of acetylation in one hexose, probably at the reducing end position. The presence of one acetyl group is also noticed by other product ions observed in the MS/MS spectrum, namely their combined loss of hexose with an acetyl group plus loss of Hexres, such as loss of $AcHex_2$ (-384 Da, ion at *m/z* at 671), loss of $AcHex_3$ (-546 Da, ion at *m/z* 509), and loss of $AcHex_4$ (-708 Da, ion at *m/z* 347) (Table 3.2.4 and Fig. 3.2.2a).

Attending to the linear ion trap mass spectrometer potentialities, which come from the ion trap capacity to perform multiple stages mass spectrometry analysis (MS^3), and in order to confirm the proposed feature of acetylated OS, the sequential fragmentation was also studied. Figure 3.2.2b shows the ESI-MS^3 experiment carried out for the ion at *m/z* 893, $[AcHex5+Na]^+$, resultant from the ion at *m/z* 1055, $[AcHex6+Na]^+$. The product ions correspond to loss of one, two and three hexoses residues, with formation of, respectively, ions at *m/z* 731 $[AcHex_4+Na]^+$, *m/z* 569 $[AcHex_3+Na]^+$ and *m/z* 407 $[AcHex_2+Na]^+$. It is also possible to see product ions formed by loss of AcHex, at *m/z* 671, and combined loss of AcHex plus one and two Hexres with formation of the ions at *m/z* 509 and 347. All the fragmentation pathways reinforce the presence of an acetylated OS.

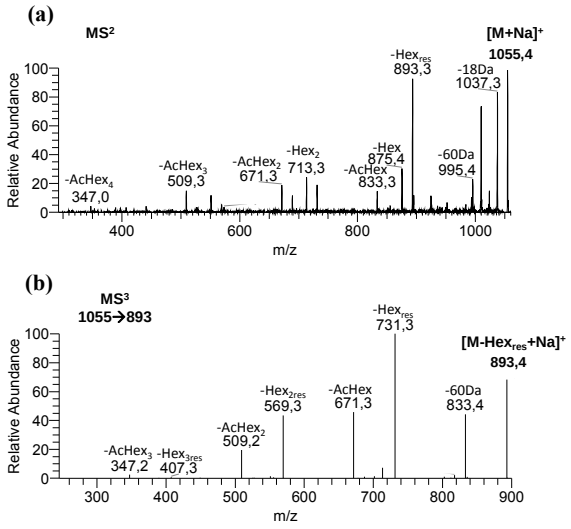

Fig. 3.2.2 *ESI-MS^n spectra of the ion at m/z 1055, attributed to $[AcHex_6+Na]^+$. (a) ESIMS/MS spectrum. (b) ESI-MS^3 spectrum of the product ion $[AcHex_5+Na]^+$ (m/z 893).*

III.2.4.2. ESI-MS^n of PentHex$_6$

The ESI-MS/MS spectrum of the ion $[PentHex_6+Na]^+$, at *m/z* 1145 (Fig. 3.2.3a) shows the product ions at *m/z* 1013, formed due to the loss of a $Pent_{res}$ (-132 Da). This fragmentation pathway shows the presence of a pentose residue in this OS. Loss of a neutral 132 Da fragment, attributed to the loss of a pentose residue, was already observed in the study of pentosyl galactomannans from coffee infusion and coffee residue (Nunes et al., 2005; Chapter III.1). Others ions, such as the ion at *m/z* 689 due to loss of $PentHex_2$ (-474 Da), the ion at *m/z* 509 due to loss of $PentHex_3$ (-636 Da), and the ion at *m/z* 347 due to loss of $PentHex_4$ (-798 Da) are also observed in the MS/MS spectrum. All these product ions confirmed the presence of one structure with a pentose residue. The losses of one, two and three hexose residues were also observed, corresponding to formation of the product ions $[PentHex_5+Na]^+$ at *m/z* 983, $[PentHex_4+Na]^+$ at *m/z* 821, and $[PentHex_3+Na]^+$ at *m/z* 659 (Table 3.2.2), as well as loss of one to three Hex, leading to the ions $[PentHex_{5res}+Na]^+$ at *m/z* 965, $[PentHex_{4res}+Na]^+$ at *m/z* 803 and $[PentHex_{3res}+Na]^+$ at *m/z* 641, all confirming the proposed structure.

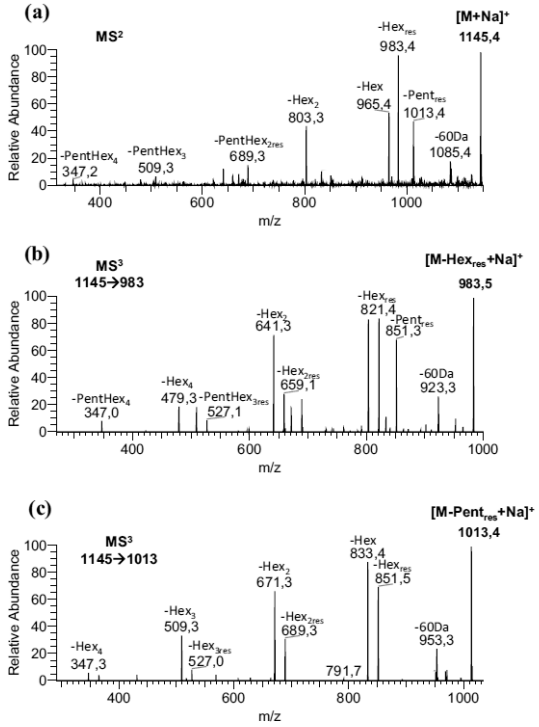

Fig. 3.2.3 *ESI-MS^n spectra the ion at m/z 1145, attributed to [PentHex$_6$+Na]$^+$. (a) ESIMS/ MS spectrum. (b) ESI-MS^3 spectrum of the product ion [PentHex$_5$+Na]$^+$ (m/z 983). (c) ESI-MS^3 spectrum of the product ion [Hex$_6$+Na]$^+$ (m/z 1013).*

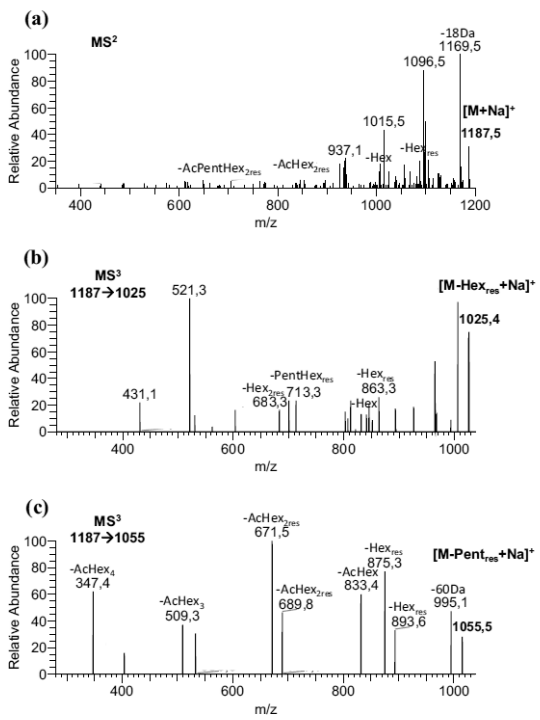

Fig. 3.2.4 *ESI-MS^n spectra the ion at m/z 1187, attributed to [PentAcHex$_6$+Na]$^+$. (a) ESIMS/ MS spectrum. (b) ESI-MS^3 spectrum of the product ion [PentAcHex$_5$+Na]$^+$ (m/z 1025). (c) ESI-MS^3 spectrum of the product ion [Hex$_6$+Na]$^+$ (m/z 1055).*

The ESI-MS^3 spectrum of the ion [PentHex$_5$+Na]$^+$, at *m/z* 983 (Fig. 3.2.3b), shows the product ions corresponding to losses of Hex residues: [PentHex$_4$+Na]$^+$ at *m/z* 821, [PentHex$_{4res}$+Na]$^+$ at *m/z* 803, [PentHex$_3$+Na]$^+$ at *m/z* 659, and [PentHex$_{3res}$+Na]$^+$ at *m/z* 641. The loss of a pentose group (-132 Da) was also observed with formation of ion [Hex$_5$+Na]$^+$ at *m/z* 851, as well as the loss of a pentose group combined with loss of hexose: loss of Pent$_{res}$Hex (-294 Da) with formation of ion [PentHex$_4$+Na]$^+$ at *m/z* 689, loss of PentHex (-312 Da) with formation of ion [Hex$_{4res}$+Na]$^+$ at *m/z* 671, loss of PentHex$_{2res}$ (-456 Da) with formation of ion [Hex$_3$+Na]$^+$ at *m/z* 527, and loss of PentHex$_2$ (-474 Da) with formation of ion [Hex$_{3res}$+Na]$^+$ at *m/z* 509 (Table 3.2.4). These product ions indicate the presence of a pentose residue group as ramification in the LBG

galactomannans structure. The ESI-MS^3 spectrum of the ion $[Hex_6+Na]^+$ at *m/z* 1013 (Fig. 3.2.3c) confirms the fragmentation of a mannan OS.

III.2.4.3. ESI-MS^n of $PentAcHex_6$

The ESI-MS/MS spectrum of the ion at *m/z* 1187, attributed to $[PentAcHex_6+Na]^+$, is shown in figure 3.2.4a. Although the low abundance of these ions, the formation of the product ion $[AcHex_6+Na]^+$ at *m/z* 1055 due to loss of $Pent_{res}$ was observed, confirming the presence of a pentose group. The formation of the product ion $[PentHex_4+Na]^+$ at *m/z* 821, due to loss of $AcHex_2$, was also identified, showing that the acetyl group was present in the galactomannans structure of LBG and it was linked to the hexose residue and not linked to the pentose residue. The product ion formed by loss of $AcPentHex_{2res}$ (*m/z* 689) and the product ions formed by loss of Hex_{res} and Hex (*m/z* 1025 and 1007, respectively) were also observed (Table 3.2.4). The presence of the acetyl groups linked to the galactomannan backbone was also confirmed by the analysis of the ESI-MS^3 spectrum of the ion $[(PentAcHex_6-Hex_{res}+Na)]^+$ at *m/z* 1025 (Fig. 3.2.4b) and the ESI-MS^3 spectrum of the ion $[(PentAcHex_6-Pent_{res}+Na)]^+$ at *m/z* 1055 (Fig. 3.2.4c and Table 3.2.4). The presence of the product ions $[Hex_{5res}+Na]^+$ at *m/z* 833, $[Hex_4+Na]^+$ at *m/z* 689, and $[Hex_{4res}+Na]^+$ at *m/z* 671, formed due to loss of AcHex, $AcHex_{2res}$, and $AcHex_2$, respectively, reinforced the presence of acetyl groups.

III.2.5. Identification of reduced permethylated arabinose containing oligosaccharides

In order to confirm the presence of arabinose residues as side chains of LBG galactomannans by an alternative independent method, GC-MS analysis of reduced and permethylated oligosaccharides formed by *endo*-β-mannanase hydrolysis were performed. The presence of a permethylated terminally-linked arabinose residue can be identified by the presence in the EI-MS spectra of a fragment ion at *m/z* 175. The extracted *m/z* 175 ion chromatogram is shown in figure 3.2.5a. The main abundant peak, with a Rt of 8.5 min showed a fragmentation pattern and Rt compatible with a permethylated ManManol. The EI-MS spectrum of this compound shows that the ion at *m/z* 175 occurs in small amount.

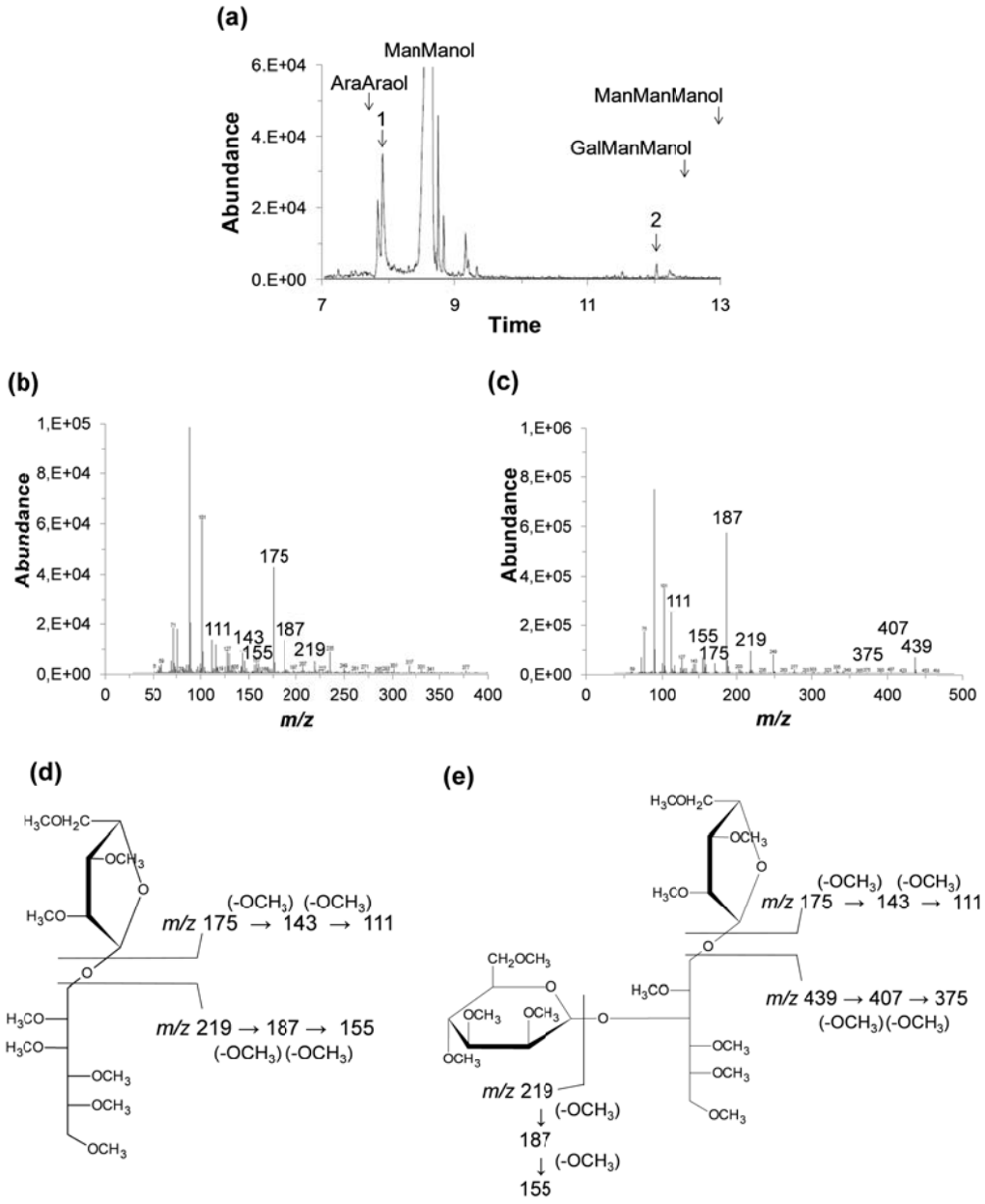

Fig. 3.2.5 *GC-MS assays of reduced and permethylated oligosaccharides formed by mannanase hydrolysis. (a) Ion extracted chromatogram using the ion at m/z 175, diagnostic for permethylated terminally-linked pentose residues, with indication of the elution time of permethylated standards of α-(1→5)-L-arabinosyl-L-arabinitol (AraAraol), β-(1→4)-D-mannosyl-D-mannitol (ManManol), β-(1→4)-D-mannosyl-[α-(1→6)-galactosyl]-D-mannitol (GalManManol), and β-(1→4)-D-di-mannosyl-D-mannitol (Man$_2$Manol). (b) EI-mass spectrum of peak 1. (c) EI-mass spectrum of peak 2. (d) Fragmentation pattern and proposed structure for peak 1, permethylated AraManol (m/z 426). (e) Fragmentation pattern and proposed structure for peak 2, permethylated AraManManol (m/z 630).*

However, because this is the most abundant compound, even using the extraction ion mode it shows the highest peak. Nevertheless, the full EI mass spectrum of peak 1 (Fig. 3.2.5b), with a Rt of 7.7 min, allows to observe a fragmentation pattern compatible with a permethylated AraManol (Fig. 3.2.5d). Also, the full EI mass spectrum of peak 2 (Fig. 3.2.5c), with a Rt of 12.0 min, allows to observe a fragmentation pattern compatible with a permethylated AraManManol (Fig. 3.2.5e). The fragmentations and retention times of these compounds confirm that arabinose is linked to the mannan backbone of LBG.

III.2.6. Concluding remarks

The detection of arabinose residues linked to the oligosaccharides formed by the specific hydrolysis of the mannan backbone of two LBG by an endo-β-mannanase, using independent methods, namely ESI-MS/MS and GC-MS, clearly shows that LBG galactomannan contains arabinose residues as side chains, linked at O-6 to the mannan backbone. ESI-MS/MS analysis also shows that LBG galactomannan contains acetyl groups linked to the mannan backbone. These structural features are similar to those previously reported to occur in coffee products resultant from coffee beans, as presented in section III.1, but with higher branching and much lower acetylation, as well as higer degree of polymerization.

RESULTS AND DISCUSSION

III.3. MASS SPECTROMETRY CHARACTERIZATION OF AN *ALOE VERA* MANNAN PRESENTING IMMUNOSTIMULATORY ACTIVITY

HIGHLIGHTS:

► Oligosaccharides (OS) were obtained from *Aloe vera* polysaccharide using *endo*-β-mannanase. ► OS were analysed by ESI-MS and MALDI-MS, by sugar and methylation analysis. ► Polysaccharide is a galactomannan with arabinose and acetyl groups as side chains. ► Acetyl groups, in an average of two per monosaccharide, were non-homogeneously distributed. ►Lower branching and some acetylation seems to promote immunostimulatory activity.

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Mass spectrometry characterization of an *Aloe vera* mannan presenting immunostimulatory activity

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Keywords: *Aloe vera*; Acetylation; Acemannan; Electrospray; MALDI; Mass spectrometry

Aloe vera acemannan is a polysaccharide composed by a backbone of β-(1→4)-linked D-mannose residues interspersed by few glucose residues, acetylated in O-2, O-3 and O-6 containing side chains constituted by O-6-linked single α-D-galactose and α-L-arabinose residues.

These structural features are rather similar to mannans from other sources, namely coffee (section III.1) and locust bean gum (section III.2). However, as *Aloe vera* acemannan, coffee mannans present immunostimulatory activity but locust bean gum does not. Also, a commercial *Aloe vera* preparation presented comparable immunostimulatory activity to that observed for coffee mannans. In order to know more about the structural features of the commercial preparation of *Aloe vera*, this preparation was submitted to a sugar and methylation analyses. To gain further insight to the structural details of the mannans, namely acetylation pattern, a specific hydrolysis with an *endo*-β-(1→4)-D-mannanase and the resulting oligosaccharides (OS) were fractionated by size exclusion chromatography and characterised by ESI-MS, ESI-MS/MS and MALDI-MS.

III.3.1. Chemical characterization of Aloe vera high molecular weight material (AV)

The sugar composition of the high molecular weight material recovered by dialysis from the commercial Aloe mucilaginous polysaccharides (AV) is shown in Table 3.3.1. The amount of carbohy-drate material was estimated to be 33.5% by sugar analysis, and was mainly composed by glucose (47%) and mannose (44%). According to glycosidic linkage composition (Table 3.3.1), this glucose occurs mainly as (1→4)-linked residues, with 6% of terminally linked glucose residues in relation to total glucose. As this polysaccharide did not colored blue in the iodine test, it could be inferred that its origin should be an excipient with a cellulosic origin. AV mannose residues were mainly (1→4)-linked (32.7% of total glycosidic link-ages observed), followed by (1→4,6)- (2.9%), and terminally linked- mannose residues (1.5%). Galactose only occurs as terminally linked residues (1.2%). This glycosidic composition confirms the presence of a galactomannan.

Table 3.3.1 *Sugar and glycosidic-linkage composition of AV.*

Linkage	% Area
T-Ara*f*	0.4
T-Ara*p*	1.4
Total Ara	1.8[a] (3)[b]
T-Man*p*	1.5
4-Man*p*	32.7
4,6-Man*p*	3.1
Total Man	37.3 (44)
T-Gal*p*	1.2 (6)
T-Glc*p*	3.5
4-Glc*p*	54.3
Total Glc	57.8 (47)

[a] Total molar percentage obtained by methylation analysis.
[b] Values in brackets are the molar percentage obtained by sugar analysis.

Table 3.3.2 compares the glycosidic linkage composition of galac-tomannans from AV and coffee, presenting immunostimulatory activity, and also of locust bean gum (LBG) galactomannan, that do not present this activity, according to already published data (Simões et al., 2009). The ratio of total Man/(1→4,6)-Man was 13, which showed an

average of 1 branched Man residue in each 13 Man residues (Table 3.3.2). This degree of branching was simi-lar to that observed for the chemically acetylated coffee residue galactomannans (Chapter III.1), but was lower than that observed for coffee infusion galactomannans, which presented a total Man/(1→4,6)-Man ratio of 28 (Nunes et al., 2005). The AV polysaccharides were, however, much less branched than LBG galactomannan that presented a total Man/(1→4,6)-Man ratio of 4 (Chapter III.2). The ratio of total Man/T-Man provides information concerning the length of the polymers, although, as observed by Nunes and Coimbra (2001) and Chapter III.1, a possible degradation of the polysaccharides during the methylation procedure could result in underestimation of the degree of polymerization of these polysaccharides (Nunes & Coimbra, 2001). According to Table 3.3.2, the ratio of total Man/T-Man of 26 for AV was comparable to those observed for coffee infusion and chemically acetylated coffee residue galactomannans and was much smaller than the 68 observed for LBG.

Table 3.3.2 *Comparison of galactomannan characteristics of Aloe vera, chemically acetylated coffee residue, coffee infusion, and locust bean gum.*

Galactomannan features	AV	Chemically acetylated coffee residue[a]	Coffee infusion[b]	LBG[c]
Total Man*p*/4,6-Man*p*	13	13	28	4
Total Man*p*/T-Man*p*	26	23	20	68
Acetylation (mol acetic acid/mol sugar)	2.08	0.98	0.08	v[d]
Ara side chains	Yes	Yes	Yes	Yes
Acetylated Man backbone	Yes	Yes	Yes	Yes
Acetylated side chains	Yes	Yes	No	No

[a] Values from Chapter III.1, [b] Values from Nunes et al. (2005), [c] Values from Chapter III.2, [d] vestigial amounts

An average of 2.08 acetyl groups was observed per sugar residue (Table 3.3.2). This value is higher than that observed for *Aloe vera* acemannans by Fogleman et al. (1992), and Hamman (2008), that determined approximately 1 acetyl group per mannose residue. The value observed of 2.08 acetyl groups is also higher than that observed for the mannans of coffee infusion (0.08, Simões et al., 2009) and LBG (below the limit of detection of the method used, Chapter III.2). Also, this acetylation content is higher than that observed in the chemically acetylated coffee mannan (0.84–0.94, Simões et al., 2009).

As observed for coffee and LBG mannans, according to Table 3.3.2, AV mannan is also composed arabinose by terminally linked arabi-nose residues (2%). However, in AV mannan, the arabinose residues occur mainly in the pyranose form (78%) instead of the furanose form found to occur in coffee and LBG.

III.3.2. Selective hydrolysis of AV by *endo*-β-(1→4)-D-mannanase

In order to obtain exclusively mannan oligosaccharides that contain the structural details and retaining the sugar and acetyl substituents of the mannan backbone for further MS studies, the hydrolysis with the *Aspergillus niger endo*-β-(1→4)-D-mannanase was performed. This selective hydrolysis was used for the study of mannans from naturally acetylated mannans present in coffee infusion (Nunes et al., 2005), from chemically acetylated mannans from coffee residue (Chapter III.1), and from several galac-tomannans (Cerqueira et al., 2011; Chapter III.2). According to the known enzymatic mechanism of *Aspergillus niger endo*-β-(1→4)-D-mannanase, this selective degradation procedure allows cleaving the galactomannan backbone between adjacent β -(1→4) linked mannose residues, allowing to obtain mannan oligosaccharides that contain structural details on the substituents of the mannan backbone (Dhawan & Kaur, 2007; Moreira & Filho, 2008). This selec-tive hydrolysis gives oligosaccharides with low molecular weight allowing their separation by size-exclusion chromatography on Biogel-P2 (Fig. 3.3.1) in fractions ready for MS analysis. This procedure also allowed discarding the contaminant glucan observed to be present in this preparation. According to figure 3.3.1, the enzymatic cleavage of AV led to the formation of OS with a higher degree of polymerization than tetrasaccharides (DP4). This contrasted with the chromatographic profile observed for coffee infusion and LBG OS, where the more abundant compounds had a DP of 2 (Chapter III.1) and between 2 and 4 (Chapter III.2), respectively. However, this profile is similar to that observed for chemically acetylated cof-fee residue (Chapter III.1), except for the fact that the small amount of DP2 or DP3 observed in coffee residue was not observed in AV.

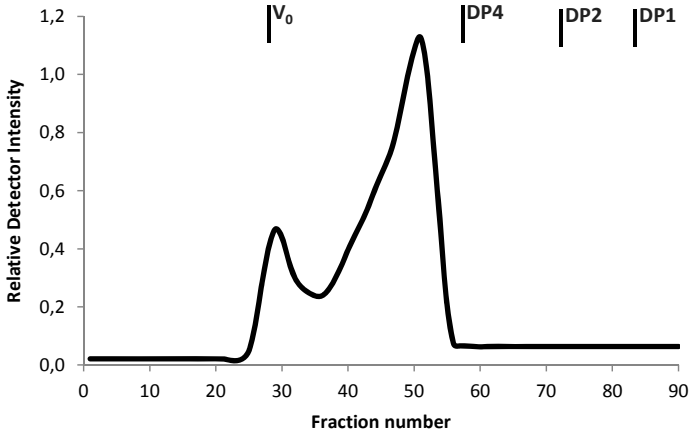

Fig. 3.3.1 *Size-exclusion chromatography on Bio-Gel P2 of the OS obtained after enzy-matic hydrolysis with an endo-β-(1→4)-D-mannanase of galactomannan from AV. V0 - void volume, DP2 and DP3 correspond to the elution volume of DP2 and DP4 standard oligosaccharides, and DP1 corresponds to the elution volume of monomers.*

III.3.3. Characterization of oligosaccharides from AV by ESI and MALDI mass spectrometry

To study the structural features of acemannan, OS obtained after selective hydrolysis with an *endo*-D-mannanase treatment and further fractionated by size exclusion chromatography, were analyzed by mass spectrometry (MS). The ions observed in the MS spectra was identified as $[M+Na]^+$ ions. Because Man, Gal, and Glc cannot be distinguished due to their isomeric nature, these hexoses are represented generically by "Hex". "Pent" represents a pentose, probably Ara, as this was the only pentose detected by sugar and methylation analysis, and "Ac" represents the acetyl groups. figure 3.3.2a shows the ESI-MS spectrum of the ions in the range of *m/z* 680 and 865, where it is possible to observe a series of $[M+Na]^+$ ions of OS composed by 4 hexose residues with acetyl groups ranging from 0 to 4 ($Ac_{0-4}Hex_4$). Also, in the range of *m/z* 800 and 1090, it is possible to observe a series of $[M+Na]^+$ ions of OS composed by 5 hexose residues with acetyl groups ranging from 0 to 5 ($Ac_{0-5}Hex_5$, Fig. 3.3.2b). The AV OS identified using electrospray ionization (ESI) are summarized in Table 3.3.3 where it is possible to observe acetylated OS in the range $Ac_{0-8}Hex_{3-6}$, including OS presenting a ratio of acetyl groups higher than one, as for example, Ac_6Hex_3, Ac_7Hex_4, and Ac_8Hex_5. This acetylation pattern shows

much more acetyl groups than those observed in coffee (Nunes et al., 2005; Chapter III.3) and LBG mannan OS (Chapter III.2). The sample that seems to approach a similar acetylation pattern is the chemically acetylated mannans from coffee residue, where it was possible to observe an $Ac_{0-3}Hex_4$ series (Chapter III.2). Figure 3.3.2c shows the occurrence, although with a small relative abundance, of $[M+Na]^+$ ions corresponding to acetylated OS with hexoses and a pentose residue, namely, $Ac_{1-4}PentHex_{1-4}$.

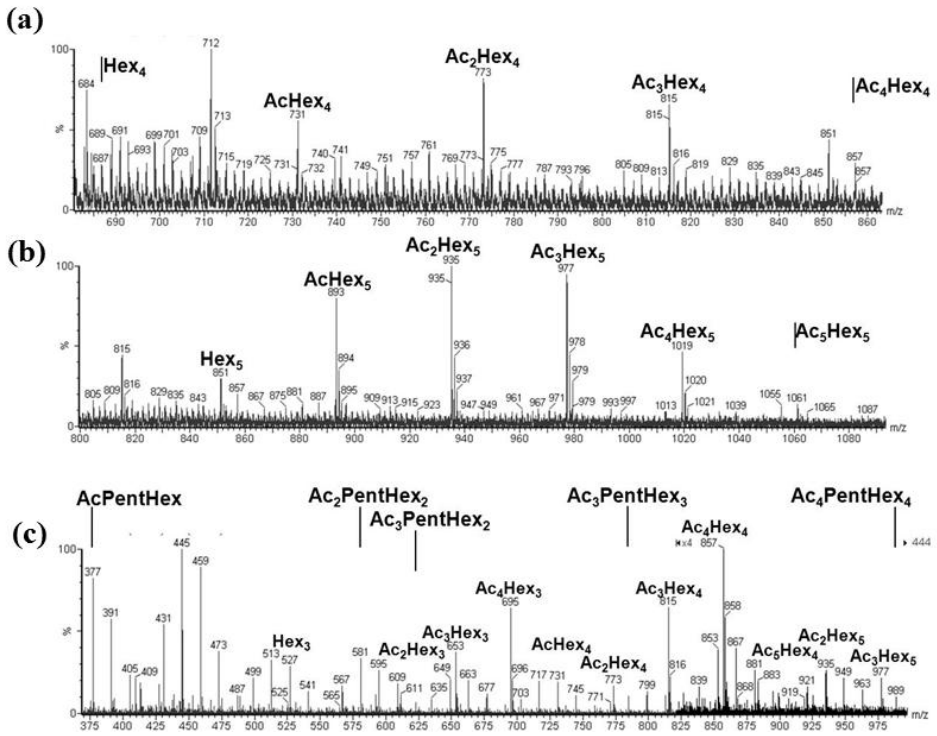

Fig. 3.3.2 *ESI-MS spectra of AV OS (a) fraction nr. 41: m/z 680-865, (b) fraction nr. 41: m/z 800-1090 and (c) fraction nr. 47: m/z 350-1500.*

Table 3.3.3 shows that it is possible of find highly acetylated OS containing a pentose, namely, $Ac_5PentHex_2$ and $Ac_6PentHex_3$. The occurrence of mannan OS containing arabinose has also been observed in coffee and LBG, although in these samples it was possible to detect non-acetylated structures, which were not found in these AV samples.

Table 3.3.3 *Oligosaccharide $[M+Na]^+$ ions from AV observed in the ESI-MS, with the identification of the m/z value and the most probable composition.*

	n	Number of acetyl groups								
		0	1	2	3	4	5	6	7	8
		m/z								
Hex_n	3	527	569	611	653	695	737	779		
	4	689	731	773	815	857	899	941	983	
	5	851	893	935	977	1019	1061	1103	1145	1187
	6	1013	1055	1097	1139	1181				
$PentHex_n$	2			581	623		707			
	3		701		785		869	911		
	4				947	989				

To evaluate the presence of OS with higher DP than those observed by ESI-MS, a MALDI-MS analysis was performed (Fig. 3.3.3 and Table 3.3.4). MALDI-MS analysis allowed to identify the $[M+Na]^+$ ions in the range of $Ac_{0-18}Pent_{0-2}Hex_{4-14}$, which included the highly acetylated OS $Ac_{15}Hex_9$, $Ac_{16}Hex_{10}$, $Ac_{15}Hex_{11}$, and $Ac_{18}Hex_{12}$. Also, MALDI-MS allowed to identify highly acetylated OS containing pentoses, as for example, $Ac_8PentHex_{11}$, and $Ac_{11}Pent_2Hex_9$. This observation confirms the high degree of acetylation even in OS of higher DP.

III.3.4. Identification of structural features in OS by tandem mass spectrometry

In order to confirm the structural features of the OS seen in the MS spectra and the presence of pentose residues and acetyl groups as structural features of this mannan, all the OS were also studied by ESI-MS/MS. With this tool, it is possible to observe product ions formed by glycosidic cleavages allowing to confirm the pres-ence of sugar residues. Also, the product ions formed by cross-ring cleavages can give information about the type of linkages (cleavage of two bonds within the sugar ring), and presence of substituting groups (Chapter III.1, Chapter III.2). To demonstrate this outcome, it will be described in detailed the product ion spectra (ESI-MS/MS) obtained for the ions $[M+Na]^+$ of an highly acetylated hexose OS $[Ac_7Hex_4+Na]^+$ (*m/z* 983), and a highly acetylated OS with a pentose residue $[Ac_5PentHex_3+Na]^+$ (*m/z* 869). The typical fragmentations observed in the MS/MS

spectra of the $[M+Na]^+$ ions of β-(1→4)-mannose oligosaccharides under ESI-MS/MS conditions are the loss of a hexose residue (Hex_{res}, -162 Da) due to glycosidic bond cleavage, the loss of water (-18 Da), and the formation of fragment ions resultant from cross-ring cleavages, $^{0.2}A_3$, with loss of 60 Da (Chapter III.1, Chapter III.2).

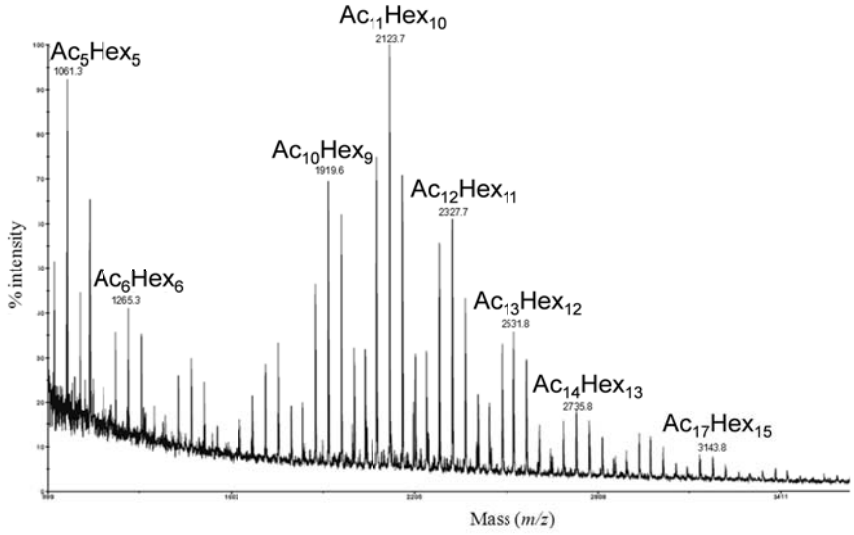

Fig. 3.3.3 *MALDI-MS spectrum of fraction nr. 31 of AV (m/z 1000-3600).*

Table 3.3.4 *Oligosaccharide $[M+Na]^+$ ions observed in the MALDI-MS spectra of the several OS fraction obtained from AV with the identification of the m/z value and the most probable composition*

$Pent_mHex_n$		Number of acetyl groups																	
m	n	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
		m/z																		
0	4				815	857														
0	5	851	893	935	977		1061													
0	6	1013	1055	1097		1181														
1	6									1481										
0	7	1175	1217	1259				1427	1469	1511	1553									
1	7									1643										
2	7												1901							
0	8							1589	1631	1673	1715	1757								
1	8									1805										
2	8											2021	2063	2105						
0	9										1877	1919	1961				2129			
1	9									1967										
2	9												2225							
0	10										2039	2081	2123			2249		2495		
1	10									2129										
0	11										2201	2243	2285	2327	2369	2411	2453			
1	11									2291										
0	12											2405	2447	2489	2531	2573	2615	2657	2699	2741
0	13												2609	2651	2693	2735	2777	2819	2861	2903
0	14															2897	2939	2981	3023	3065

III.3.4.1. MS/MS of Ac_7Hex_4

The ESI-MS/MS spectrum of $[Ac_7Hex_4+Na]^+$ (*m/z* 983) ions (Fig. 3.3.4) showed the presence of ions at *m/z* 821 ($[Ac_7Hex_3+Na]^+$), formed due to glycosidic bond cleavage with loss of one Hex_{res} (-162 Da). The product ions at *m/z* 965, due to loss of water (-18 Da), and at *m/z* 923, resulting from crossring cleavage (-60 Da) were also observed, in accordance to the characteristic fragmentation of β-(1→4)-linked hexose oligosaccharides. This ion at *m/z* 923 could be resulting also due to loss of an acetyl group (CH_3COOH) (Reis et al., 2005; Chapter III.1). This spectrum shows also the product ions at *m/z* 761, 719, and 677, due to loss of AcHex, Ac_2Hex, Ac_3Hex, respectively, and also the product ions at *m/z* 557, 515, 473, and 431, resultant from loss of $Ac_{2-5}Hex_2$. These fragmentation

pathways confirm the presence of acetyl groups linked to Hex in the OS structures, reaching the number of three acetyl groups per hexose. Assuming the β- (1→4)-linkage, the occurrence of Ac_3Hex and Ac_5Hex_2 shows that all hexose carbons, C_2, C_3, and C_6, should be acetylated (Manna & McAnalley, 1993; Talmadge et al., 2004). The loss of 162 Da allows to infer that this OS does not contain any acetyl group at the non-reducing end residue. This confirms that this OS is resultant from the *endo*-β-(1→4)-D-mannanase hydrolysis, as for the enzymatic hydrolysis to occur a non-substituted mannose residue should be present forming the glycosidic linkage to be cleaved, thus leading to the formation of OS with a non-substituted mannose residue at the new formed non-reducing end.

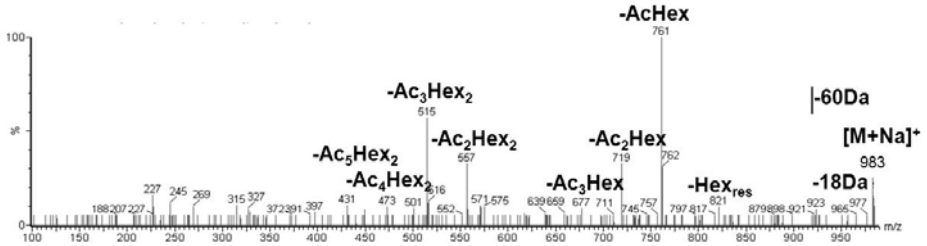

Fig. 3.3.4 *ESI-MS/MS spectrum of the $[M+Na]^+$ ion of Ac_7Hex_4 (m/z 983) obtained from AV.*

III.3.4.2. MS/MS of $Ac_5PentHex_3$

The ESI-MS/MS spectrum of the $[Ac_5PentHex_3+Na]^+$ (*m/z* 869) ion (Fig. 3.3.5) shows the product ions at *m/z* 719 due to loss of Pent (-150 Da), correspondent to $[Ac_5Hex_{3res}+Na]^+$, confirming the presence of a pentose group in this OS. It was also observed the characteristic loss of a hexose residue (-Hex_{res}; -162 Da), corresponding to the ion observed at *m/z* 707, $[Ac_5PentHex_2+Na]^+$. The ions at *m/z* 695 and 653 correspond to $[Ac_4Hex_3+Na]^+$ and $[Ac_3Hex_3+Na]^+$, respectively, formed due to the loss of $Ac_{1\text{-}2}Pent_{res}$. The ions at *m/z* 677, 635, and 593 correspond to the ions $[Ac_4Hex_{3res}+Na]^+$, $[Ac_3Hex_{3res}+Na]^+$, and $[Ac_2Hex_{3res}+Na]^+$, respectively, formed due to loss of $Ac_{1\text{-}3}Pent$, showing that pentose groups of the mannan understudy were also acetylated. The loss of Ac_3Pent shows that all pentose carbons are acetylated. This is a surprising result, not expect to be obtained in acemannan accord-ing to the available literature (Talmadge et al., 2004). However, a similar pattern has been observed when the mannans from coffee residue were chemically acetylated, showing the occurrence of a higher extent of

acetylation than that observed in the natural poly-mer, as well as acetylated pentoses (Chapter III.1). Also, the absence in all spectra of the loss of 102 Da, attributed to a combined loss of cross ring fragmentation $^{0.2}A_3$ with an acetyl group linked, is comparable with the product obtained by the chemical acetylation of coffee residue mannan. However, the OS from coffee infusion mannans showed this loss of 102 Da, confirming the location of the acetyl group at the C2 of the hexose of the naturally acetylated product (Chapter III.1).

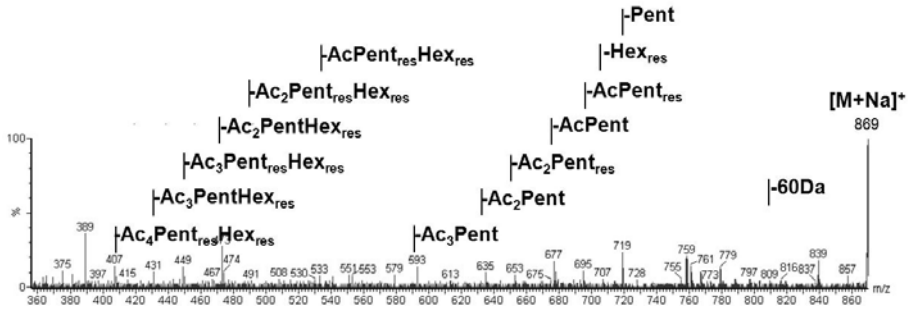

Fig. 3.3.5 *ESI-MS/MS spectrum of the $[M+Na]^+$ ion of $Ac_5PentHex_3$ (m/z 869) obtained from AV.*

III.3.5. Concluding remarks

The results obtained by mass spectrometry of *Aloe vera* acemannan structural features allowed to observe for the first time the presence of arabinose residues in this structure, as previously was observed for coffee (section III.1) and LBG (section III.2) galactomannans. This mannan contained, on average, 2 acetyl groups per sugar unit, which is the double of that reported in literature for this polysaccharide. Also, it is more acetylated than the coffee mannans that showed similar immunostimulatory activity. Furthermore, in this polysaccharide, acetyl groups were non-homogeneously distributed, as it was possible to observe oligosaccharides with three acetyl groups per hexose unit, as well as non-acetylated hexose OS presenting DPs that varied between 3 and 7. In addition, pentose side chains were also identified as well as acetylated pentose residues. These pentose side chains can be attributed to arabinose residues, as identified in sugar and methylation analyses. The results obtained and the comparison with the other galactomannans from coffee infusion and residue, and LBG allow to infer that the lower branching, shorter chains, and some acetylation are the structural features that seem to promote the immunostimulatory activity of these polysaccharides.

CHAPTER IV

EXTRACTION AND STRUCTURE

OF COFFEE RESIDUE POLYSACCHARIDES

RESULTS AND DISCUSSION

IV.1. EXTRACTION OF COFFEE BY-PRODUCT POLYSACCHARIDES BY ROASTING PRE-TREATMENT AND HEATED ALKALI SOLUTIONS – – EFFECT ON EXTRACTABILITY AND STRUCTURE

HIGHLIGHTS:

► Roasting at 160ºC followed by 4 M NaOH treatments at 20, 60, and 120 ºC improved the extractability of galactomannans from coffee by-product. ► A total of 56% of galactomannans were extracted. ► No degradation concerning the degrees of polymerization and branching and acetylation pattern was observed. ► A total of 54% of coffee by-product arabinogalactans were also extracted.

Extraction of coffee by-product polysaccharides by roasting pre-treatment and heated alkali solutions – effect on extractability and structure

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Keywords: Coffee residue; galactomannans; arabinogalactans; roasting; mass spectrometry; SEM.

The coffee residuet obtained after the preparation of the brew is very rich in polysaccharides, namely, galactomannans and arabinogalactans, which are polymers that can be used as dietary fiber and present immunostimulatory activity. Considering the huge amount of coffee by-product produced all over the world, the reutilization of this by-product by its application as food ingredients is very promising. However, the yields of extraction of these polysaccharides tend to be very low, namely the galactomannans. Based on the observation that the yield of galactomannans extracted from the ground coffee to the brew increase when the coffee is roasted, in this study, with the aim of increasing the yield of the extraction of these polysaccharides, the coffee by-product was roasted and then extracted with hot water and alkali solutions. The different extracts obtained were characterised by sugar and linkage analysis. Also, *endo*-β-manannase degradation and fractionation of the digests through BioGel P2 was used to determine the structural features of the galactomannans by mass spectrometry. The coffee residues obtained after alkali extraction were analysed by scanning electron microscopy.

IV.1.1. Yield of extraction of polysaccharides from roasted coffee by-product and characterisation of fractions extracted with hot water and 4 M NaOH at 20ºC

Espresso coffee by-product sample used was composed mainly by mannose (46%), galactose (27%), glucose (20%), and arabinose (7%) (Table 4.1.1).

Table 4.1.1 *Sugars composition of initial coffee by-product and fractions from the extraction with 4M NaOH at different temperatures of roasted coffee by-product.*

	Yield of extraction (%)	Sugar composition (%mol)				Total Sugar (%)
		Ara	Man	Gal	Glc	
Initial sample of coffee by-product		7	46	27	20	60.0
T160_H_2O_90	1.4	14	33	51	2	44.3
T160_NaOH_20sn	7.8	14	24	61	1	63.2
T160_NaOH_20ppt	9.8	4	73	21	2	65.9
T160_sn IR_20	4.6	17	14	68	1	42.1
T160_IR_20		5	52	19	24	75.0
T160_NaOH_60sn	2.3	16	12	71	1	51.0
T160_NaOH_60ppt	6.5	2	86	11	1	81.0
T160_IR_60		5	44	20	31	71.7
T160_NaOH_120sn	2.6	17	15	67	1	71.2
T160_NaOH_120ppt	5.7	2	89	7	2	80.1
T160_IR_120	20.0	6	45	24	25	57.1
T220_H_2O_90	0.2	8	51	37	4	21.2
T220_NaOH_20sn	9.4	6	47	45	2	52.6
T220_NaOH_20ppt	5.3	2	76	11	11	70.0
T220_ IR_20		4	42	24	30	15.0
T220_NaOH_60sn	4.4	6	59	31	4	48.6
T220_NaOH_60ppt	0.8	2	54	9	35	33.7
T220_IR_60	53.5	4	30	18	48	12.4

The higher abundance of mannose allows to infer that the galactomannans are the major polysaccharides present, accounting for approximately 50% of total carbohydrates. Performing a hydrolysis with 2 M of H_2SO_4 at 100ºC during 2.5h, the total carbohydrates were observed to account for 60% of the dry weight material of coffee by product. This value was higher than the 45% estimated when 1 M of H_2SO_4 was used under the same conditions. However, the same relative proportions of sugars have been observed in the

two hydrolysis conditions (data not shown). These observations allow to infer that the total polysaccharides can be underestimated when 1 M of H_2SO_4 is used.

Under the experimental conditions described for the roasting of coffee by-product at 160 ºC, during the first 10 min the temperature was below 160 ºC due to the water evaporation. After 1 h, the material still retained 34% of water, but after 2 h, a weight loss of 1% was observed. For the roasting pre-treatment at 220 ºC, after 1 h, the material still retained 11% of water. When the pre-treatment was prolonged for more 30 min, a weight loss of 35% was obtained. In order to evaluate the effect of these pre-treatments on the extent of coffee residue polysaccharides extractability, the two roasted residues were extracted with hot water at 90 ºC during 1 h and then the solubilised material was dialysed. For the roasting at 160 ºC, the amount of polymeric material recovered with hot water only accounted for 1.4 % of total residue (Table 4.1.1), that were composed by 44% of sugars. The main sugar residue was galactose (51%), mannose accounting for only 33% of total sugars. This shows that the arabinogalactans were the polysaccharides preferably extracted under these conditions. For the roasting at 220 ºC, the hot water polymeric extracted material accounted only for 0.2 % of total residue and was composed only by 21% of sugars, which was lower than that observed for the 160 ºC pre-treatment. However, in this roasting conditions, the main sugar residue was mannose (51%), accounting galactose only for 37% of total sugars, showing that under these conditions, the majority of the arabinogalactans, are not extracted.

Taking into account that coffee by-product galactomannans are soluble and extractable in 4 M NaOH solutions (Simões et al., 2009), the residues left after the hot water extraction of the roasted material were suspended in a 4 M NaOH solution at room temperature during 2 h. Upon dialysis of the neutralized material, in both experiments, it was obtained a fraction of material that was insoluble in cold water (NaOH_20ppt) and a fraction of material that was soluble (NaOH_20sn). The material roasted at 160 ºC was mainly recovered in the precipitate (9.8%) whereas the material roasted at 220 ºC was mainly recovered in the supernatant (9.4%). The supernatants were, in both cases, richer in galactose (61 and 45%, respectively) and poorer in mannose (24 and 47%) than the precipitates (21 and 11% for galactose, and 73 and 76% for mannose, respectively). As the supernatants were also rich in arabinose, it can be inferred that they contain mainly

arabinogalactans, thus indicating that arabinogalactans were removed by the alkali and remained soluble upon neutralisation. However, the extraction from the 220ºC residue also contained higher amount of soluble mannose, allowing to infer an increase of extraction of alkaline extracted water soluble galactomannans.

The insoluble residues obtained after the alkali extraction were suspended in water, neutralised, and dialysed. The material that was solubilised during dialysis was recovered in the supernatant after centrifugation (T160_snIR_20), separated from the insoluble material (T160_IR_20). For the 160ºC roast, the snIR_20 fraction accounted for 4.6 % of the starting material, on a dry basis, whereas 42% were sugars. The main sugar residue of this fraction was galactose (67%), accounting arabinose for 17% and mannose for only 14% of total sugars, allowing to infer the presence of arabinogalactans as the main polysaccharide. For the 220ºC roast, no material was obtained in the snIR_20 fraction. The T160_IR_20 fraction obtained from the 160ºC experiments, was mainly composed by mannose (52%) followed by glucose (24%), galactose (19%) and arabinose (5%), in a 75% of total sugar. This composition shows that a large proportion of galactomannans still remained in residue. A comparable sugars composition to T160_IR_20 was obtained for the T220_IR_20 residue obtained from the 220ºC experiments, although this material contained a lower amount of total sugars (15%).

Figure 4.1.1 shows the Scanning Electronic Microscopy (SEM) analysis of the initial coffee by-product (Fig. 4.1.1a), and of the residues left after the alkali extraction of roasted coffee by-product at 160ºC (T160_IR_20, Fig. 4.1.1b) and 220ºC (T220_IR_20, Fig. 4.1.1c). Comparing with the initial sample, the T160_IR_20 residue showed the appearance of more defined and hollowed structures, possibly the cell walls. This may indicate that although some material was solubilised by the alkali reagent, some still remain in the matrix, confirming the results obtained by the sugar analysis of the residue. However, in T220_IR_20, these better defined structures are not so well visible, showing possibly the degradation of the matrix resultant from a roasting at this high temperature. This is accordance with the low sugars content (15%) of this residue.

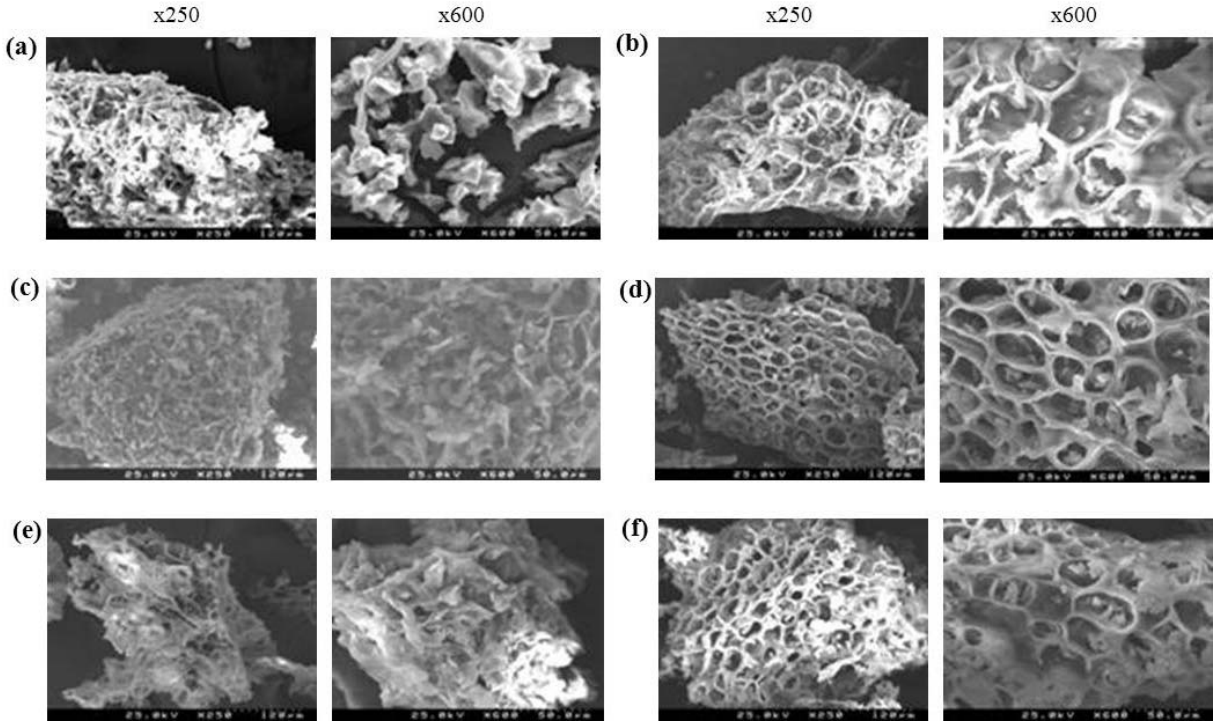

Fig. 4.1.1 *Scanning electronic microscopy (SEM) images at x250 and x600 of coffee by-products.* ***(a)*** *Initial coffee sample* ***(b)*** *T160_IR_20* ***(c)*** *T220_IR_20* ***(d)*** *T160_IR_60* ***(e)*** *T220_IR_60* ***(f)*** *T160_IR_120.*

IV.1.2. Yield of extraction of polysaccharides from roasted coffee by-product and characterisation of fractions extracted with 4 M NaOH at 60 and 120ºC

In order to further extract the coffee residue polysaccharides that still remaining in the residues T160_IR_20 and T220_IR_20, a 4 M NaOH extraction was performed at 60ºC (NaOH60). As observed for the material extracted at 20ºC, Table 4.1.1 shows that the material roasted at 160 ºC and extracted with NaOH at 60ºC was mainly recovered as a precipitate (T160_NaOH_60ppt, 6.5% of yield of extraction relative to the initial coffee by-product sample) whereas the material roasted at 220 ºC was mainly recovered in the supernatant (T220_NaOH_60sn, 4.4%). Also as observed for the material extracted at 20ºC, the supernatant of T160 was richer in galactose (71%) and poorer in mannose (12%) than the precipitates (11 and 86%, respectively), allowing to infer the occurrence of arabinogalactans as the main polysaccharides. However, both T220_NaOH_60sn and ppt,

were richer in mannose than galactose, showing that, under these higher roasting conditions, the galactomannans are the main polysaccharide extracted. Considering that fraction T160_NaOH_60ppt was rich in sugars (81%), was the most abundant one, and was the richest in mannose, these conditions seem to be relevant for extraction of galactomannans from coffee residue. However, in contrast, fraction T220_NaOH_60ppt was not so rich in sugars, the yield was low, and was not rich in mannose. Also, the determination of 35% of glucose in this precipitated fraction shows that degraded cellulose should be also extracted under these extreme conditions.

The insoluble residues obtained after the alkali extraction ate 60ºC were suspended in water, neutralised, and dialysed, giving origin to the T160_IR_60 and T220_IR_60 residues. No material was recovered in the dialysis supernatant. For the 160ºC roast, the T160_IR_60 fraction was mainly composed by mannose (44%) followed by glucose (31%), galactose (20%) and arabinose (5%), in a 72% of total sugar. This composition shows that a large proportion of galactomannans still remained in residue. On the contrary, the sugars composition obtained for the T220_RI_60 residue was poor in sugars (12%), and the main sugar residue was glucose (48%), accounting mannose only for 30% (Table 4.1.1). These results show that no relevant carbohydrate material was any longer present in T220_RI_60 residue.

To perform quantitative analysis of insoluble residues, the material should be previously treated with 72% sulphuric acid solution for a complete soaking of the polysaccharides (Selvendran et al., 1979). However, when the sugars are loosely bound to the matrix, this treatment can be discarded. In order to observe if the galactomannans and cellulose were loosely bound in the cell wall matrix due to the roasting process, explaining the occurrence of the high amount of glucose in the T220_NaOH_60 fraction, the sugars of the two residues, T160_RI_60 and T220_RI_60, were analysed with and without this treatment. Figure 4.1.2 shows the extra amount of mannose and galactose obtained when the 72% sulphuric acid treatment was performed. The amount of mannose tightly bound to the matrix accounted for 40% in T160_RI_60 and 25% in T220_RI_60. Also, the amount of cellulosic glucose was higher in the T160_RI_60 (90%) than in T220_RI_60 (70%). These results show that the roasting process of the coffee residue promotes degradation of the polysaccharides tightly bound to the cell wall matrix.

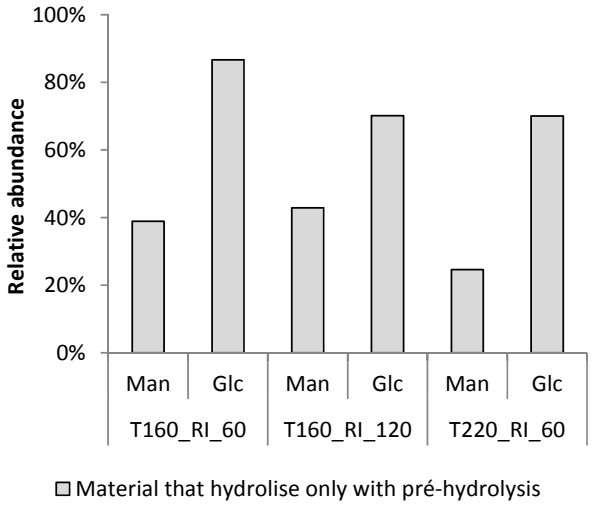

Fig. 4.1.2 *Sugar residues content with pre hydrolysis procedure of T160_RI_60, T160_RI_120 and T220_RI_60.*

SEM analysis of T160_IR_60 (Fig. 4.1.1d) showed the appearance of even better defined cell walls of hollowed cells than those observed in T160_IR_20 (Fig. 4.1.1b), resultant from the removal of all surrounding material. However, in the SEM images of T220_IR_60 residue (Fig. 4.1.1e) it was not observed the well defined cell-wall matrix visible in the T160_IR_60 residue, possibly due to the extensive degradation of the material. These observations are in accordance with the high sugar content in T160_IR_60 residue and the lower sugar content of T220_IR_60 residue (Table 4.1.1).

In order to further extract the coffee residue polysaccharides still remaining in the T160_RI_60 residue, a 4 M NaOH extraction was performed at 120ºC. The total yield of the extract was 8.3% of the initial coffee residue. The material that precipitated upon neutralisation and recovered by dialysis (T160_NaOH_120 ppt) accounted for 5.7% of the initial residue. It was composed by mannose (89%), galactose (7%), glucose (2%), and arabinose (2%), in a 80% of total sugars, which are characteristics of a pure galactomannan. The material that remained in the supernatant upon neutralisation and dialysis (T160_NaOH_120 sn) was composed mainly by galactose (66%) and arabinose (17%), in a fraction containing 71% of sugars, showing the presence of an arabinogalactan. The residue left (T160_RI_120) contained 45% of mannose. Taking into account the

amount of yield of the extracts and their content in mannose, it can be estimated that only 16% of the mannose constituent of the initial residue still remains in the final residue.

SEM analysis of T160_IR_120 residue (Fig. 4.1.1f) showed defined cell walls of hollowed cells comparable to those observed in T160_IR_60 residue (Fig. 4.1.1d), although some compacted material seems to occur. According to figure 4.1.2, the amount of mannose and glucose tightly bound to the matrix of T160_RI_120 residue accounted for 40 and 70%, respectively. These results show that although degradation of the polysaccharides tightly bound to the cell wall matrix was higher than that observed in T160_RI_60 residue, it was not so high as that observed in T220_RI_60 residue.

IV.1.3. Glycosidic linkage of galactomannans from roasted coffee by-product extracted with 4 M NaOH at 20, 60 and 120ºC

The results of methylation analysis of the fractions rich in mannose, recovered as precipitates upon extraction of roasted coffee by-product by 4 M NaOH, are shown in Table 4.1.2. The predominance of the (1→4)-linked mannose residues, and the presence of (1→4,6)- and terminally-linked mannose residues, as well as terminally-linked galactose residues, confirm the presence of galactomannans.

Table 4.1.2 *Glycosidic-linkage composition of coffee by-product galactomannans submitted to a roasting procedure at 160ºC and 220ºC, extracted with 4M NaOH solutions at different temperatures: 20ºC, 60ºC and 120ºC.*

	Without Roasting pre-treatment NaOH_20ppt[a]	T160_NaOH_ppt			T220_NaOH_ppt	
		20	60	120	20	60
Linkage	Area %					
T-Ara*f*	4.8	0.4	0.6	0.1	0.1	0.2
5- Ara*f*	2.9	0.5	1.3	0.5	0.0	1.1
T-Man*p*	3.0	7.5	2.9	1.7	3.9	2.3
4-Man*p*	60.8	70.6	84.6	90.1	77.3	42.2
4,6-Man*p*	5.4	1.0	0.9	1.5	0.0	0.9
T-Gal*p*	6.4	3.5	1.3	0.8	0.0	1.0
3-Gal*p*	8.5	9.7	0.7	1.1	2.1	1.4
3,6-Gal*p*	6.2	1.2	0.0	0.3	0.0	0.0
4-Glc*p*	1.2	5.5	7.6	4.0	16.6	48.6
Total Man*p*/T-Man*p*	23	11	30	56	21	20
(4,6-Man*p* /Total Man*p*) x100	7.8	1.3	1.0	1.6	0.0	2.1

[a] Values from Chapter III.1

The ratio of total Man/terminally-linked Man gives a direct indication of the polymer size. In the samples submitted to a roasting procedure at 160ºC and then extracted with NaOH at 20ºC, a value of 11 was obtained. This is lower than the value of 23 obtained previously for the galactomannan-rich extracts obtained from coffee residue extracted with 4 M NaOH at 20ºC without the prior roasting pre-treatment (Chapter III.2). However, when a following extraction was performed at 60ºC by the same strength alkali solution, the galactomannans obtained presented a total Man/terminally-linked Man of 30, and this ratio further increased to 56 for the galactomannans obtained when a third extraction was performed at 120ºC. In the samples submitted to a roasting procedure at 220ºC, the ratio of total Man/terminally-linked Man was similar for the two extractions performed (21 and 20 for the extractions at 20 and 60ºC).

The ratio (1→4,6)-Man/Total Man allows to infer the degree of branching of the galactomannans. All galactomannan-rich fractions extracted from the coffee by-product

roasted at 160ºC had a similar degree of branching, showing an average of 1 branched residue in 100 Man residues, which is a very low degree of branching when compared with the coffee residue galactomannans previously extracted without the roasting pre-treatment (7.8). This lower branching of the galactomannans obtained from roasted residues show that the roasting promoted the debranching of galactomannans, as previously stated by (Nunes & Coimbra, 2002a; 2002b). This observation is reinforced by the data obtained for the galactomannans extracted from the coffee by-product roasted at 220ºC, where in one fraction (T220_NaOH_20ppt) no (1→4,6)-Man neither terminally-linked galactose were detected. In all samples, it was possible to detect (1→3)-Gal, (1→3,6)-Gal, and (1→5)-Ara, which are diagnostic linkages for the presence of arabinogalactans. The relative content of these residues tend to decrease when the temperature of the alkali reagent used for extraction increased.

IV.1.4. Yields of extractions of polysaccharides

Based on the extraction yield of the different fractions, and content of the different sugars and glycosidic-linkage composition, it was possible to estimate the relative amount of galactomannans and arabinogalactans extracted (Table 4.1.3). For the estimation of the galactomannans, it was assumed that the mannose obtained was all from galactomannans and that the branching of the galactomannans in coffee residue is, on average, 8% (Chapter III.1). Due to the lower content of glucose, arabinose and acetyl groups in these galactomannans, their contribution were not considered. Based on these assumptions, the amount of galactomannans in each extract was estimated by the sum of the amount of mannose plus 8% of the total amount of mannose, accounting for the galactose content.

Table 4.1.3 *Extraction yield (%) of coffee by-product galactomannans and arabinogalactans extracted with 4M NaOH at different temperatures (20 ºC, 60ºC and 120ºC) from a coffee by-product submitted to a roasting pre-treatment at 160ºC and 220ºC.*

	NaOH extraction temperature	Extraction yield (%)	
		Galactomannans	Arabinogalactans
T160	20ºC	23	38
	60ºC	17	7
	120ºC	16	9
	Total	56	54
T220	20ºC	18	14
	60ºC	5	4
	Total	23	18

Previous results by Simões et al.(2009) reported that the alkaline extraction of coffee by-product galactomannans without any pre-treatment of roasting provided an extraction yield of 20%. The extraction of coffee by-product galactomannans after a roasting pre-treatment at 160ºC (T160) using the same extraction conditions allowed to obtain an extraction yield of 23% of these polysaccharides. The extraction of galactomannans from coffee by-product with NaOH solutions at 60ºC extracted more 17% and the extraction with NaOH at 120ºC, extracted extra 16%. These results showed that the roasting pre-treatment at 160ºC followed by successive NaOH extractions with increasing temperatures (20, 60, and 120 ºC) improved the extractability of galactomannans from coffee by-product to an aggregate of 56%. In case of the roasting pre-treatment of coffee by-product at 220ºC (T220) followed by NaOH extraction at 20ºC, the galactomannans extraction yield was only 18%. The extraction with NaOH solutions at 60ºC allowed to obtain more 5%, showing that these conditions were too extreme to recover the galactomannans.

For the estimation of the arabinogalactans, it was assumed that all galactose, except an amount corresponding to 8% of total mannose, is component of the arabinogalactans. Also, it was assumed that all arabinose in fractions is from the arabinogalactans.

The extraction arabinogalactans from roasted coffee by-product at 160ºC with the NaOH solution at 20 ºC allowed an yield of 38%, which is lower than the value of 43%

reported for the extraction of arabinogalactans from coffee by-product without any roasting pre-treatment (Simões et al., 2009). Also, the successive extractions of the residue with NaOH solutions at 60 ºC and 120 ºC only allowed to obtain extra 7 and 9%, respectively, in a total of 54% (Table 4.1.3). These results show that, contrarily to what was observed for galactomannans, the roasting of the coffee by-product does not allow to improve the extraction yield of the arabinogalactans. Also, the use of heated alkali solutions does not allow to obtain much more polysaccharides. This was confirmed in the case of the roasting pre-treatment at 220ºC, where the NaOH solution at 20 ºC only allowed to extract 14% of the arabinogalactans, and the extraction at 60ºC only allowed to obtain an extra 4%.

IV.1.5. Selective hydrolysis by an *endo*-β-(1→4)-D-mannanase

In order to evaluate the structural features of the roasted coffee by-product galactomannans obtained from different roasting procedures and extraction conditions, they were selectively hydrolysed with an *endo*-β-(1→4)-D-mannanase. The hydrolysis of the β-(1→4) linked mannan backbone is hindered by the presence of substituted residues, allowing to obtain galactomannan oligosaccharides that contain structural details on the substituents of the galactomannan backbone (Dhawan & Kaur, 2007; Moreira & Filho, 2008; Chapter III.1, Chapter III.2). The oligosaccharides obtained were fractioned by size-exclusion.

The size-exclusion chromatograms of the mixture of OS obtained after hydrolysis of the coffee by-product galactomannans submitted to roasting procedure at 160ºC, which was extracted with 4 M NaOH at 20ºC, and at 60ºC and at 120ºC (Fig. 4.1.3a). The hydrolysis of the galactomannan obtained after extraction with at 20ºC and at 60ºC, originated mainly trisaccharides (DP3) while in the case of the hydrolysis of galactomannan extracted at 120ºC, the major oligosaccharides observed were disaccharides (DP2). In these three extractions a very small amount of oligosaccharides of a higher degree of polymerisation were also observed. As the enzyme was able to completely degrade the galactomannans, it can be inferred that they keep their β-(1→4) linked mannan characteristics. The size-exclusion chromatogram of the coffee by-product galactomannans submitted to roasting procedure at 220ºC and extracted with NaOH at 20 ºC (Fig. 4.1.3b)

had a similar pattern to those roasted at 160ºC, showing that significant backbone modification occurred in these polysaccharides. However, the galactomannans extracted at 60ºC showed higher molecular weight oligosaccharides, allowing to infer that these polysaccharides have been modified in their backbone.

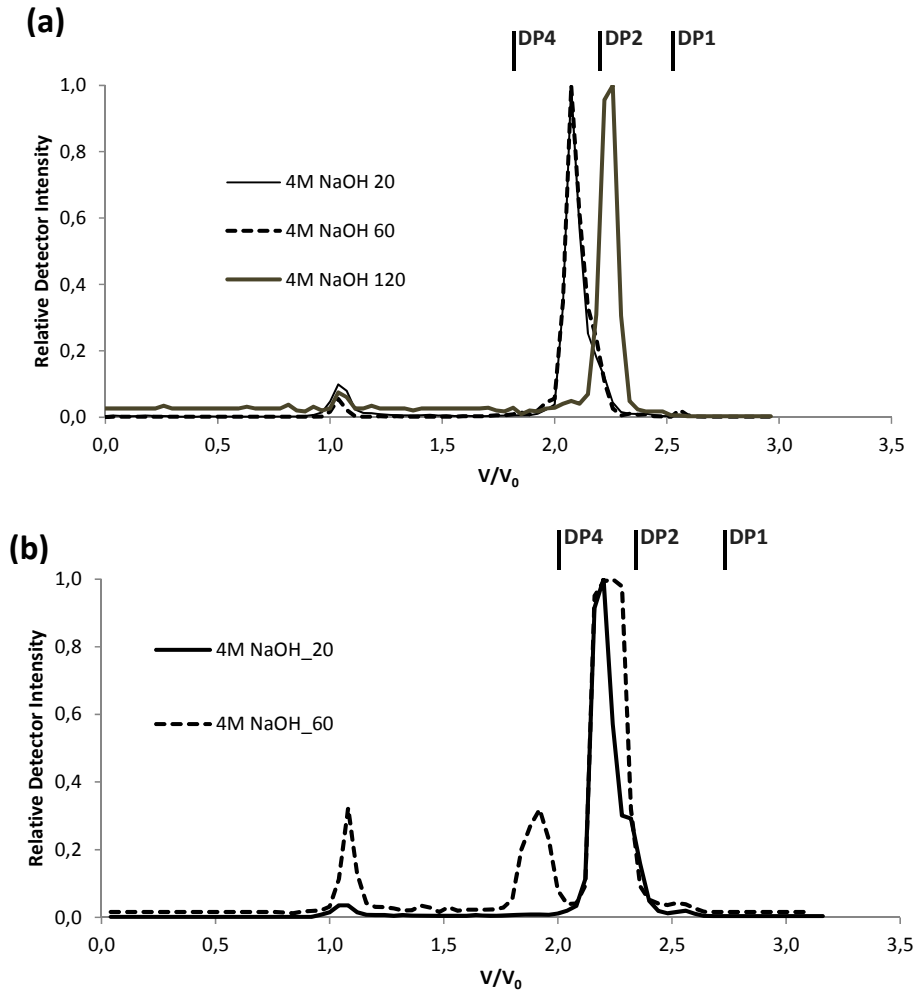

Fig. 4.1.3 *Size-exclusion chromatography on Bio-Gel P2 of the OS obtained after enzymatic hydrolysis with an endo-β-D-mannanase of galactomannan obtained from coffee by-product* ***(a)*** *T160_NaOH_20ppt, T160_NaOH_600ppt and T160_NaOH_120ppt* ***(b)*** *T220_NaOH_20ppt and T220_NaOH_600ppt . DP4 and DP2 correspond to the elution volume of DP4 and DP2 standard oligosaccharides, and DP1 corresponds to the elution volume of monomers.*

IV.1.6. Characterization of galactomannans by mass spectrometry

In order to analyse in detail the structural features of the galactomannans extracted from the roasted coffee by-product, different fractions obtained by the cleavage with an *endo*-β-mannanase were analysed by mass spectrometry with electrospray ionization (ESI-MS). The oligosaccharides present in the collected fractions were identified in ESI-MS spectra as $[M + Na]^+$ ions and it were obtained information about the molecular weight of these oligosaccharides. The OS identified in the MS spectra were confirmed and more information about the structure was obtained by ESI-MS/MS analysis. ''Hex" represents a hexose, as the *m/z* of Man, Gal or Glc is the same; ''Pent" represents a pentose, Ara because this was the only pentose detected by sugar analysis and methylation analysis, and ''Ac" represents the acetyl groups.

The ESI-MS/MS spectrum of the ion at *m/z* 497, identified as $[PentHex_2+Na]^+$ (Fig. 4.1.4a), shows the ions at *m/z* 365, *m/z* 335, *m/z* 317, and *m/z* 203, that can be attributed to losses of a pentose residue ($Pent_{res}$), a hexose residue (Hex_{res}), a hexose, and a Pent, respectively. These results allow to observe the occurrence of a pentose residue in the galactomannan structure (Fig. 4.1.4b), showing that even when the galactomannans are submitted to an extensive roasting treatment at 160 ºC, and extracted with strong alkali solutions at temperatures up to 120ºC, the galactomannans maintain their branching characteristics. These structural features were also observed in the OS obtained from the roasting procedure at 220ºC, although in very small amount (data not shown).

The product ion spectrum (ESI-MS/MS) obtained for the ion at *m/z* 407, identified as $[AcHex_2+Na]^+$ is present in all the three alkali galactomannan-rich samples extracted from the coffee by-product roasted at 160 ºC (Fig. 4.1.4c). The presence of product ions at *m/z* 245 and 227, which can be attributed to the ions $[AcHex + Na]^+$ and $[AcHex_{res} + Na]^+$, can be resultant from loss of one Hex_{res} (162 Da), and one Hex (180 Da), respectively, from the ion $[AcHex_2+Na]^+$. Also, the presence of one acetyl group in this OS is also noticed by the abundant ion at *m/z* 347, resultant of loss of $-CH_3COOH$ (60 Da) (Reis et al., 2005; Chapter III.1). The occurrence of the ion at *m/z* 305, resultant from loss of 102 Da, attributed to a $^{0,2}A_3$ cross ring fragmentation containing the acetyl group at O-2 position (Nunes et al., 2005), shows that the acetyl group is linked to the O-2 of the hexose residue

of the reducing end terminal (Fig. 4.1.4a and 4.1.4c). The observed fragmentation allowed to infer the presence of acetyl groups in roasting coffee by-product, similarly as was observed from coffee by-product without roasting procedure (Chapter III.1). Acetyl groups have been described to occur in green coffee galactomannans (Oosterveld et al., 2004), in coffee infusion from roasted coffee beans (Nunes et al., 2005) but not in coffee residue. These results show that even when the galactomannans are submitted to an extensive roasting treatment at 160 ºC, and extracted with strong alkali solutions at temperatures up to 120ºC, the characteristic acetyl groups of galactomannans are still observed. The presence of acetyl groups were also observed in the OS obtained from the roasting procedure at 220ºC, although in very small amount (data not shown), confirming their resistance to temperature and alkali reagents.

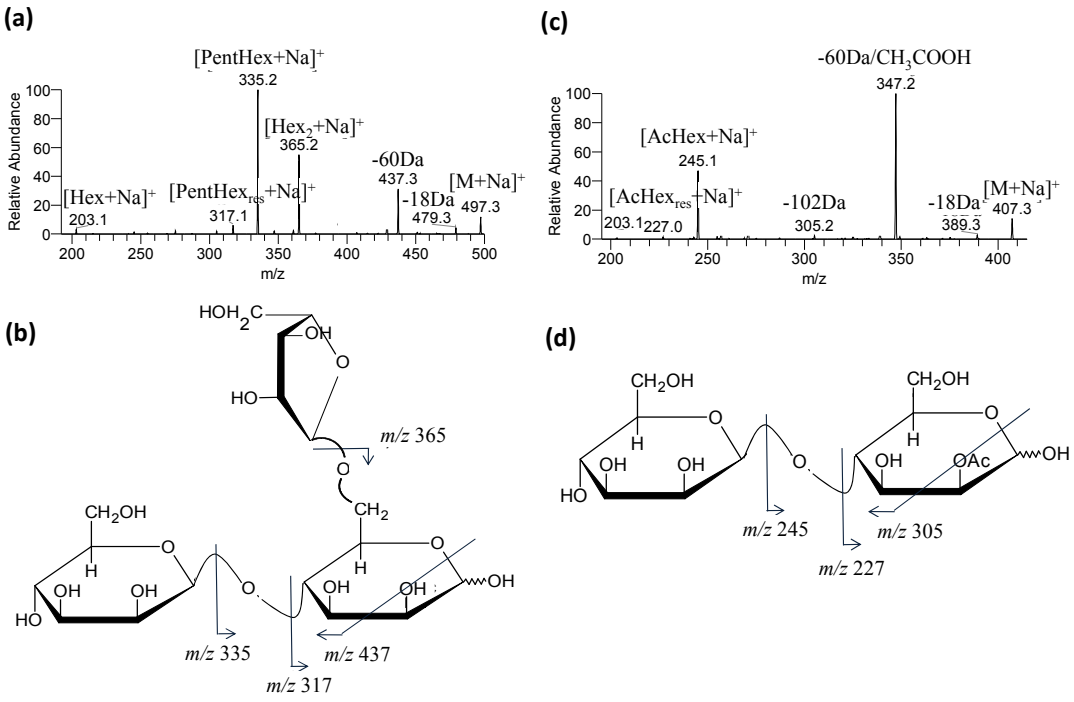

Fig. 4.1.4 *Tandem mass spectrometry assays of oligosaccharides formed by endo-β-mannanase.* ***(a)*** *ESI-MS/MS spectrum ion at m/z 497, attributed to [PentHex$_2$+Na]$^+$ and* ***(b)*** *correspondent fragmentation pattern and proposed structure.* ***(c)*** *ESI-MS/MS spectrum ion at m/z 407, attributed to [AcHex$_2$+Na]$^+$ and* ***(d)*** *correspondent fragmentation pattern and proposed structure.*

IV.1.7. Concluding remarks

The selective hydrolysis with *endo*-β-mannanase of roasted coffee by-product mannans followed by the separation of the released oligosaccharides by size exclusion chromatography, and their analysis by ESI-MS/MS shows that the roasting process at 160 ºC followed by 4 M NaOH extractions at 20 ºC, 60 ºC, and 120 ºC applied to coffee by-product improves the extractability of galactomannans without their apparent degradation, preserving their β-(1→4)-Man backbone, Gal and Ara side chains, and acetylation.

The extreme roasting process, at 220 ºC, promotes the loss of a large extent of mannans. However, their structural features and even the small details like acetylation and Gal and Ara side chains are kept.

The roasting process at 160 ºC complemented with extractions with NaOH at 20, 60, and 120 ºC could be used to successfully extract galactomannans, valorising the coffee by-product as a source of galactomannans. This procedure, although suitable for galactomannans, does not improve the extractability of the arabinogalactans.

EXTRACTION AND STRUCTURE

OF COFFEE RESIDUE POLYSACCHARIDES

RESULTS AND DISCUSSION

IV.2. THERMOGRAVIMETRIC STABILITY OF COFFEE RESIDUE POLYSACCHARIDES: GALACTOMANNANS AND ARABINOGALACTANS

HIGHLIGHTS:

► Galactomannans did not loss mass at T ≤ 200 ºC during 3h. ► Roast promoted transglycosylation, depolymerization, and isomerisation of galactomannans. ► Roast of water insoluble galactomannans promoted their solubility. ► Arabinogalactans did not loss mass at T ≤ 180 ºC during 3h. ► The thermal decomposition of arabinogalactans was higher than that of galactomannans.

Thermal stability of coffee by-product polysaccharides: galactomannans and arabinogalactans

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Keywords: Thermogravimetry; roasting; coffee residue; mannans; galactans; activation energy of thermal decomposition; mass spectrometry.

In order to better understand the thermal stability of coffee by-product galactomannans and arabinogalactans and the reactions that can occur upon roasting, a set of related polysaccharides were thermally treated at different temperatures: 160, 180, 200, 220, and 240 ºC. For this experimental design long term isothermal exposures, up to 3 h were considered. The activation energies of thermal degradation were determined as well as their thermal behavior profiles. The resultant products were analyzed according to the sugars and linkage composition and also by electrospray mass spectrometry.

IV.2.1. Polysaccharides thermal behaviour

Figure 4.2.1a shows the plot of the thermogravimetic analysis, performed at 10 ºC/min until 600 ºC, of a dried coffee residue, obtained as a by-product from espresso coffee preparation. According to the first derivative plot (Fig. 4.2.1b), the major weight losses occurred at 309 ºC (56 %), 439 ºC (19 %), and 497 ºC (17 %). The total sample mass was lost at 510 ºC, allowing to infer that the coffee by-product did not contain significant amount of minerals. When the same experiment was performed using a coffee galactomannan-rich fraction, obtained from coffee residue by strong alkali extraction and precipitation upon neutralization (Chapter IV.1.), the major mass loss was observed at 323 ºC, resulting in a 48% mass loss, which is a higher temperature and lower mass loss than that observed for the coffee residue. This galactomannan-rich fraction showed to be more resistant to weight loss at higher temperatures than the coffee residue. At 540 ºC all material was lost. Comparing the thermal behavior of coffee galactomannan with locust been galactomannan, where the later contains a Man/Gal ratio of 3.5, which is lower than that of the 12.7 verified for coffee galactomannan, it is possible to observe that the galactomannan from locust bean gum starts degrading first than coffee galactomannans. This observation allows to infer that the galactomannan from coffee by-product was less heat labile than locust bean gum galactomannan, possibly due to the lower degree of branching, resulting in a higher inter-chain hydrogen bonding and a material with higher crystallinity. The locust bean gum galactomannans presented a higher decrease in mass loss (72 %) than coffee galactomannans (48 %) at a narrow temperature gap (317 and 323 ºC, respectively) showing that the coffee galactomannan was less homogeneous than the locust bean gum. Both galactomannan samples showed a higher temperature for the highest mass loss when compared with the coffee residue. Comparing the thermal degradation of all material analyzed with the cellulose thermal degradation, it was observed that cellulose had the highest mass loss (86%) as well as the highest temperature of degradation (343 ºC), confirming the higher resistance of this unbranched and highly polymerized polysaccharide. As the coffee by-product sample is composed by a mixture of galactomannans, cellulose, and arabinogalactans, the difference observed may be due to the presence of the arabinogalactans, which has been reported to be a more heat labile polysaccharide (Redgwell et al., 2002b). In order to analyze the thermal behavior of a pure arabinogalactan, thermogravimetric analysis of the polysaccharide from Gum Arabic was

performed (Fig. 4.2.1a). The major mass loss was observed at 315 ºC, which was lower when compared to the galactomannans. These results show that the thermal behavior of coffee by-product was modulated by the presence of both galactomannans, cellulose, and arabinogalactans. The galactomannans and cellulose confer to the coffee structure more resistance to the thermal treatment than the arabinogalactans. According to figure 4.2.1a and b, it was observed that the galactomannans were degraded at temperatures approaching 300 ºC. However, in long term exposures they should degrade at lower temperatures. In order to evaluate the long term thermal resistance of coffee galactomannans, experiments were performed using isothermals at 160, 180, 200, 220, and 240 ºC during 3 h. figure 4.2.1c presents the different isothermal weight loss curves of a coffee galactomannan with time. It is possible to observe an initial mass loss stage from 0 to 10 min, which can be attributed to the loss of adsorbed water. This loss was approximately 8% for all isotherms except for 220 ºC, that was slightly lower (6%). The rate of mass loss ranged between 0.120 mg/min at 160 ºC and 0.096 mg/min at 240 ºC. After this mass loss, no variation was observed for the experiments performed at temperatures below 200 ºC during the 3 h. However, mass losses were observed at 220 ºC (4 %) and 240 ºC (10 %), ranging from 0.0028 mg/min to 0.0083 mg/min, respectively. These results show that the galactomannan was thermally stable at temperatures lower or equal to 200 ºC during long term exposures up to 3 h. Taking into account that the coffee roasting process is usually performed at temperatures around 200 ºC, it is expectable that during the roasting of the coffee beans the galactomannans keep their thermal stability. The thermal behavior of the galactomannans at 220 ºC shows that thermally resistant compounds may be formed, decreasing the rate of mass loss for the shorter period of temperature exposure but increasing for long term degradation.

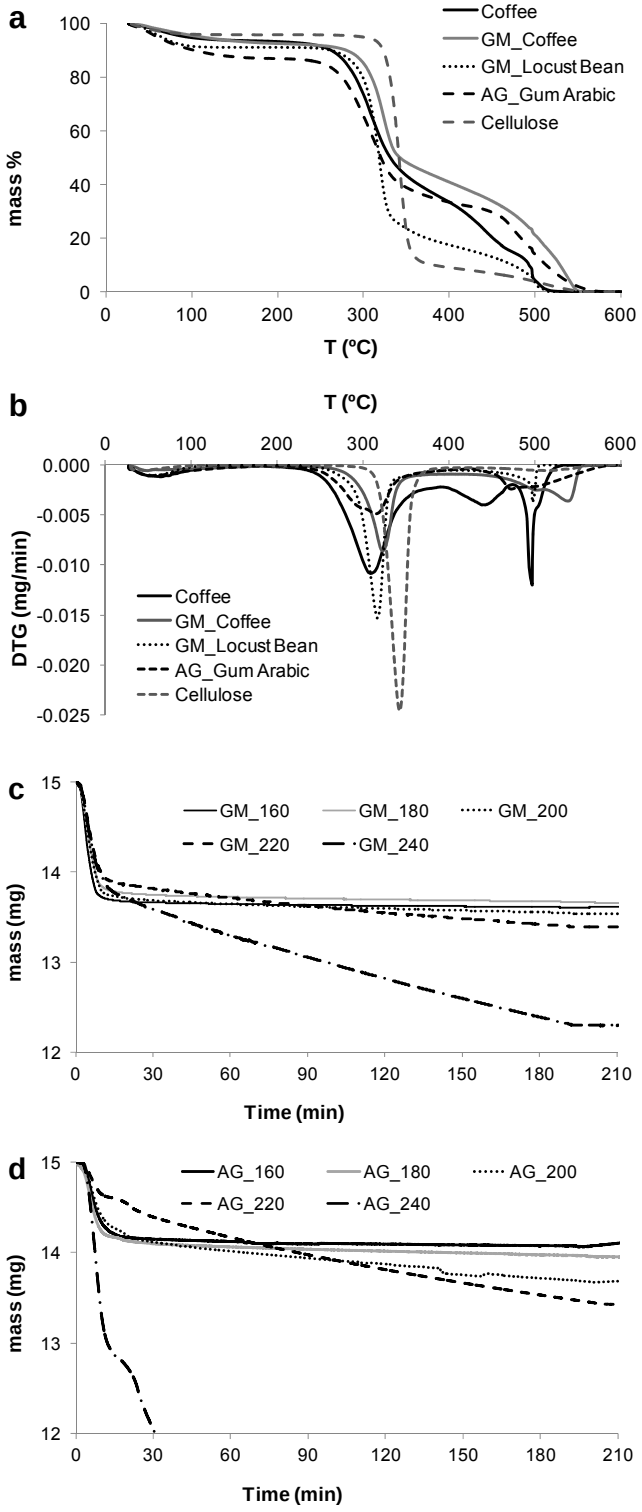

Fig.4.2.1 (a) *Thermograms heating rate of 10 °C/min and* ***(b)*** *respective 1st derivative of the weight loss curve of samples: Coffee, GM_Coffee, GM_Locust Bean and AG_Gum Arabic.*
Thermograms at different isothermal (160, 180, 200, 220 and 240 ºC) of ***(c)*** *GM_Coffee* ***(d)*** *AG_Coffee.*

IV.2.2. Activation energy of thermal decomposition

The acceleration of thermal degradation can be quantitatively represented by determining the apparent activation energy of thermal decomposition (Ea) for different stages of degradation of the material. The Ea of thermal decomposition of coffee galactomannans and arabinogalactans rich-fraction as well as coffee by-product, locust bean gum galactomannan, cellulose, and arabinogalactan from gum arabic, was determined through the thermograms obtained. The activation energies were calculated by the integral method of Broido (Broido, 1969). The Broido equation is ln ln(1/y) = (Ea/R).1/T + constant, where y is defined by the relationship y = (Wt-Wf)/ (Wi-Wf). Wt denotes the weight of the sample at any time while Wi and Wf stand for its initial and final weights, respectively. T is the absolute temperature in Kelvin, recorded on the thermogram. Thus, a plot of ln ln(1/y) vs. 1/T gives a straight line whose slope is related to the apparent energy of activation. The plot ln ln(1/y) vs 1000/T was used to calculate the apparent activation energies, in kJ/mol, based on the rates of mass loss when the temperature was continuously increased at a constant rate of 10 ºC/min (Fig. 4.2.2a).

The Ea of thermal decomposition of coffee galactomannan was 138 kJ/mol, which is a value lower than the 209 kJ/mol determined for the LBG galactomannan. This difference can be explained by the higher molecular weight of LBG when compared to the coffee galactomannan (Chapter III.2)., Kok et al. (1999) determined an Ea value for LBG of 110 kJ/mol but at 121ºC and no references to coffee galactomannans activation energy of thermal decomposition were found. It is difficult to compare the results because, as Vasile et al. (2011) discussed, depending upon the heating rate and the experimental conditions used to examine the kinetics, different values can easily emerge. The Ea values obtained for galactomannans were however lower than the Ea determined for cellulose (325 kJ/mol),wich was also in accordance with the literature. In literature, some different values for the activation energy of thermal decomposition for cellulose were found, such as 195-213 kJ/mol (Varhegyi et al., 1997) and 200-240 kJ/mol (Font et al., 1991). In contrast, the Ea value determined for gum arabic arabinogalactan (71 kJ/mol) was much lower, allowing to infer a higher heat lability of this polysaccharide. In literature, the Ea for gum arabic presents also lower values than cellulose, varying between 125 - 155 kJ/mol (Cozic

et al., 2009) and 122 kJ/mol (Zohuriaan & Shokrolahi, 2004). These values are high than the ones obtained in our work probably also due to the different origin of the samples. The coffee by-product presented also a lower Ea value (67 kJ/mol), showing that the arabinogalactans may have influence

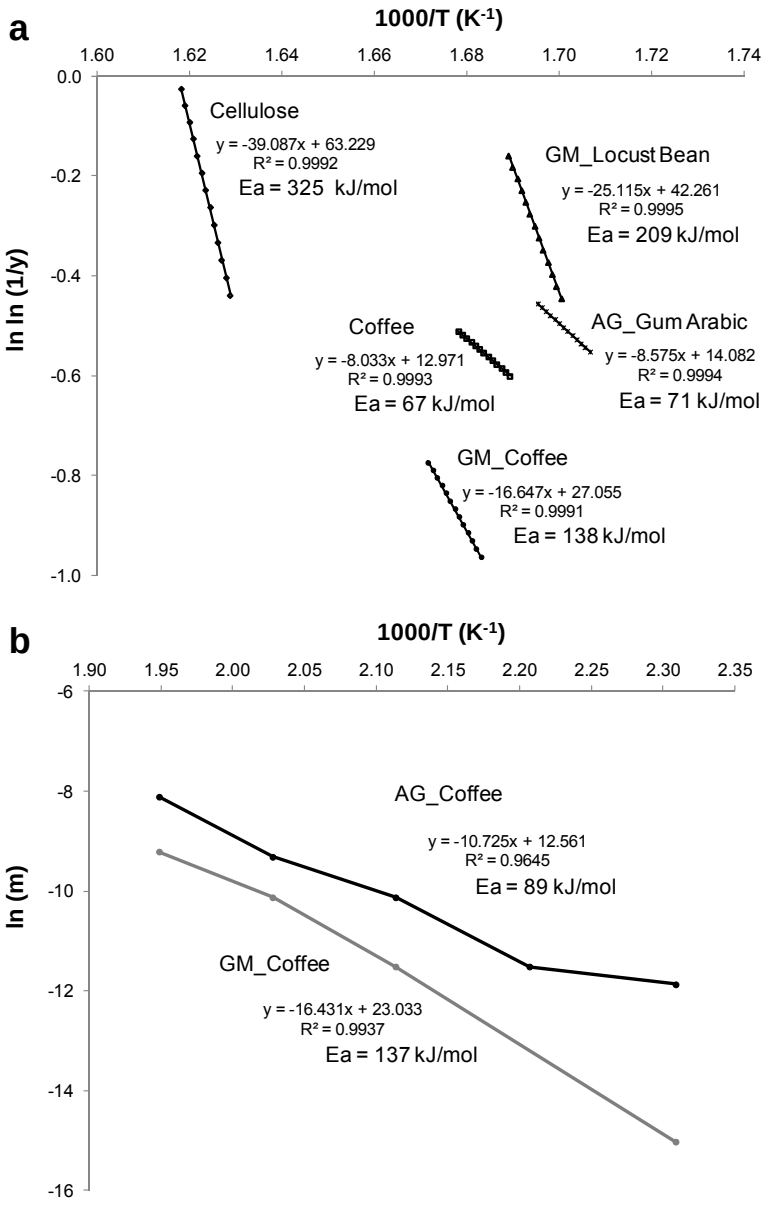

Fig. 4.2.2 *Determination of activation energy of thermal decomposition.*

Using the thermograms obtained by the isothermal thermogravimetric analysis (Fig. 4.2.1a and 4.2.1b), it was possible also to determine the Ea by using the plot ln(m) vs.1/T

of the equivalent equation: ln (m) =-(Ea/R)•1/T + constant, where m is the slope obtained in different thermograms, T is the temperature documented on the thermogram, in Kelvin, an R is the gas constant (8.314x10-3 $kJK^{-1}mol^{-1}$). The Ea of thermal decomposition estimated for coffee galactomannan was 137 kJ/mol, confirming the Ea value previously obtained. In this experiment it was also possible to estimate the Ea of a coffee arabinogalactan-rich fraction, showing an activation energy of thermal decomposition of 94 kJ/mol, which was lower than that determined for the galactomannan. This difference allows to conclude that the coffee galactomannans were more thermal resistant than coffee arabinogalactans. Also, comparing with the gum arabic arabinogalactan determined in the previous experiment, the Ea of coffee arabinogalactan was slightly higher, possibly due to the roasting process that could confer to the polysaccharide left higher thermal resistance.

IV.2.3. Structural changes of galactomannans after thermal treatments

IV.2.3.1 Coffee galactomannans

In order to analyse the changes on the structural features of galactomannans due to the thermal procedures, a linkage analysis of galactomannan samples submitted to different roasting conditions were performed (Table 4.2.1). The predominance of the (1→4)-linked mannose residues (63-90 mol%), and the presence of (1→4,6)- and terminally-linked mannose residues (2-10 and 2-12 mol%, respectively), as well as terminally-linked galactose residues, checks the presence of galactomannans. The relative abundance of (1→4)-linked mannose residues decreased as the temperature of roasting increased whereas the (1→4,6)- and terminally-linked mannose residues increased with the increase of the roasting temperature. As the terminally-linked Man gives a direct indication of the polymer size, it can be inferred that the polysaccharide degree of polymerization decreased with the increase of the roasting temperatures up to 240 ºC. The relative abundance of the (1→4,6)-Man residues are usually used to infer the degree of branching of the galactomannans (Nunes & Coimbra, 2001). However, when methylation analysis is used, the presence of 1,6-anhydromannose residues that can be formed at the reducing end of manno-oligosaccharides when they are roasted (Moreira et al., 2011), forming the same

partially methylated derivative diagnostic of (1→4,6)-Man residues should also be taken into consideration.

In the galactomannan rich-fraction without any thermal treatment (GM), a relative abundance of 1.5 mol% was observed for (1→4,6)-Man whereas an increase to 5.6, 6.0, 7.2, 9.0, and 10.1 mol% was observed for (1→4,6)-Man for GM_160, GM_180, GM_200, GM_220, and GM_240. As the roasting of coffee promotes the debranching of galactomannans (Nunes & Coimbra, 2002a and 2002b), it is possible that the depolymerization of the galactomannans will allow the formation of reducing end sugar units that, in these roasting conditions, promoted the formation of 1,6-anhydromannose. According to Moreira et al. (2011), the roasting also promotes the formation of new linkages and polymerization. An increase of the formation of (1→6)-linked (0-6 mol%) and, in a small extent, (1→2)-linked Man residues (0.0-0.3 mol%) was observed with the increase of the temperature of roasting. These new linkages were more abundant in GM_220 and GM_240. According to Moreira et al. (2011) they may be resultant from depolymerization together with the formation of new linkages (transglycosylation). Also, it was observed the increase of (1→4)-linked Glc along the increment of the roasting temperatures (4-10 mol%). Although (1→4)-linked Glc is part of the structure of coffee galactomannans (Nunes et al., 2005), these linkages may also occur due to isomerization reactions upon roasting at the reducing terminal of galactomannans (Nunes et al., 2006a).

In all samples, it was possible to detect small amounts of (1→3)-Gal, (1→3,6)-Gal, and (1→5)-Ara, which are diagnostic linkages for the presence of arabinogalactans. It is possible that this small portion of arabinogalactan structural features associated to the galactomannans is resultant from polymerization reaction between arabinogalactans and galactomannans (Nunes & Coimbra, 2007).

Table 4.2.1 *Glycosidic-linkage composition (%mol) of coffee by-product galactomannans rich fraction submitted to different thermal treatment at 160, 180, 200, 220 and 240 ºC, and submitted to a ashing furnace assay at 240ºC.*

Linkage	GM	GM_160	GM_180	GM_200	GM_220	GM_240	GM_240_F	
							HMWM	LMWM
T-Ara*f*	0.1	0.4	0.4	0.2	0.3	0.1	0.0	0.0
5-Ara*f*	0.5	1.0	2.0	1.5	1.0	1.8	0.0	0.0
T-Man*p*	1.7	3.8	4.5	6.3	9.5	11.9	4.1	49.2
2-Man*p*	0.0	0.0	0.0	0.0	0.1	0.3	0.0	0.0
4-Man*p*	90.1	80.1	74.4	73.6	68.0	63.0	93.8	42.1
6-Man*p*	0.0	0.2	0.5	1.0	2.6	5.7	0.0	2.3
4,6-Man*p*	1.5	5.6	6.0	7.2	9.0	10.1	2.1	2.9
T-Gal*p*	0.8	1.5	1.7	1.7	1.6	1.1	0.0	0.6
3-Gal*p*	1.1	1.4	1.0	1.5	1.5	0.7	0.0	0.2
6-Gal*p*	0.0	1.5	0.6	0.6	1.4	0.7	0.0	0.2
3,6-Gal*p*	0.3	0.6	1.1	0.8	0.8	0.7	0.0	0.2
4-Glc*p*	4.0	4.0	8.5	6.8	7.0	9.9	0.0	0.0

IV.2.3.2 Model studies with manno-oligosaccharides

In order to understand the effect of a long term roasting on the β-(1→4)-linked mannose residues and confirm our previous results, a model study with small OS were used. Mannotriose (Man_3) and mannotetraose (Man_4) oligosaccharides were roasted at 160 ºC and 200 ºC done and analyzed by methylation analysis (Table 4.2.2) and mass spectrometry with electrospray ionization (ESI-MS). No weight loss was observed in these assays, although the colour of the oligosaccharides turned from white to pale yellow at 160 ºC and to brittle caramel dark brown at 200 ºC.

Table 4.2.2 *Glycosidic-linkage composition (mol%) of mannotriose and mannotetraose with and without thermal treatment at 160 ºC and 200 ºC.*

Linkage	Man_3	Man_3_160	Man_3_200	Man_4	Man_4_160	Man_4_200
T-Man*p*	33.7	46.4	52.1	29.1	31.4	49.2
2-Man*p*	0.0	0.0	0.1	0.0	0.0	0.1
4-Man*p*	58.1	47.8	18.4	63.3	60.7	28.7
4,6-Man*p*	3.5	1.6	7.9	2.7	1.9	5.3
6-Man*p*	0.4	0.4	17.2	0.7	0.7	7.6
T-Gal*p*	3.1	2.5	0.8	2.4	2.2	1.3
4-Glc*p*	1.0	1.2	3.5	1.4	2.3	7.3

The glycosidic linkage composition of roasted Man_3 and Man_4 roasted at 160 ºC and 200 ºC, when compared with the non-roasted oligosaccharides, showed a decrease in the relative abundance of (1→4)-linked mannose residues from 58 to 48 and 18 mol%, respectively, for Man_3 and from 63 to 61 and 29 mol%, respectively, for Man_4. On the contrary, the terminally-linked mannose residues increased with the increase of the roasting temperature from 34 to 46 and 52 mol% for Man_3 and from 29 to 31 and 49 mol%, respectively, for Man_4. These results show that the cleavage of (1→4)-linked residues and the appearance of higher relative proportion of terminally-linked residues, possibly resultant of depolymerization, are promoted in higher extent with the increase of the roasting temperature. It can also be noticed that the oligosaccharides roasted at 200 ºC presented high relative amounts of (1→6)-linked mannose residues, both as (1→6)- or as (1→4,6)-Man residues. For Man_3, the proportion of these glycosidic linkages increased from 0.4 and 3.5 to 17 and 8 mol%, respectively, and for Man_4 the increase was from 0.7 and 2.7 to 8 and 5 mol%. Also, it was observed the increase of (1→4)-linked Glc from 1.0 and 1.4 mol% in Man_3 and Man_4, respectively, to 3.5 and 7.3 mol% after roasting at 200 ºC for 2 h. These results are in accordance with the formation of new glycosidic linkages by transglycosilation, the formation of 1,6-anhydromannose residues, and the occurrence of isomerization reactions similar to those observed for the coffee galactomannans submitted to the different roasting temperatures.

In order to confirm the differences observed for the oligosaccharides roasted at 160 and 200 ºC, they were analyzed by ESI-MS. Figure 4.2.3 shows the spectra of the oligosaccharides obtained from mannotriose after the roasting at 160 ºC (Fig. 4.2.3a) and at 200 ºC (Fig. 4.2.3b). Whereas at 160 ºC the major ions were observed at *m/z* 527, corresponding to $[Hex_3+Na]^+$, and at *m/z* 1031, corresponding to $[2Hex_3+Na]^+$, at 200 ºC, the major ions were observed at *m/z* 347, 509, 671, and 833, attributed to the anhydrohexose-containing ions $[Hex_{1-4}AnHex+Na]^+$. Also, although in lower abundance, this spectrum shows the ions at *m/z* 995, 1157, and 1319, attributed to the ions $[Hex_{5-7}AnHex+Na]^+$. Also, in this spectrum the ion at *m/z* 527 had an abundance similar to the ion at *m/z* 365 corresponding to $[Hex_2+Na]^+$, showing that the formation of the polymerized anhydrous forms is also accompanied with depolymerization reactions. These reactions occur at 200 ºC but not at 160 ºC even at a long exposure of 2 h.

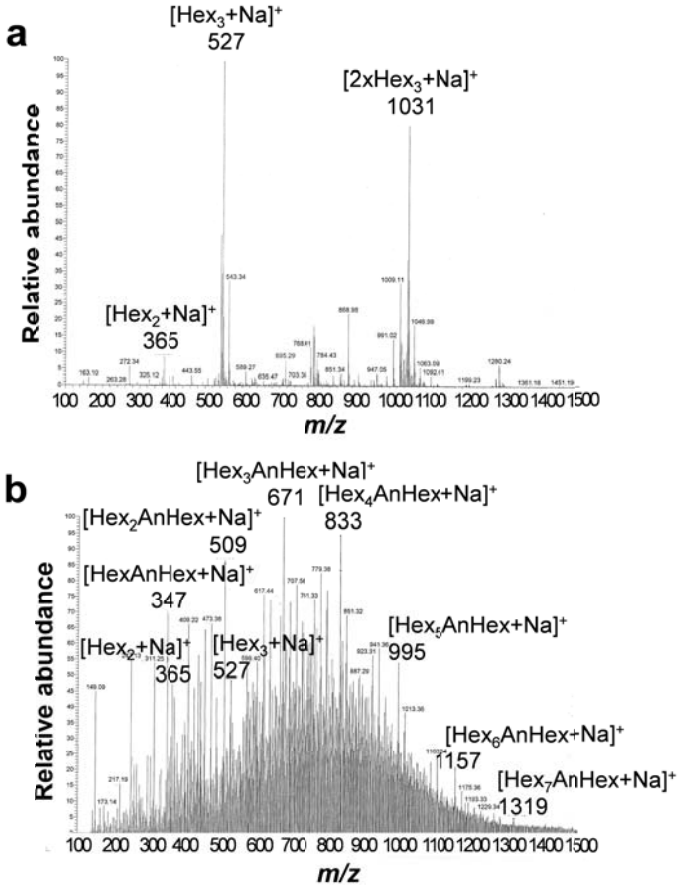

Fig. 4.2.3 *ESI-MS spectra of (a) Man_3 submitted to a roasting procedure at 160 ºC (b) Man_3 submitted to a roasting procedure at 200 ºC.*

IV.2.3.3.Low molecular weight material

During the thermal treatment polymerization and depolymerisation occurs, so it could be important analyse the low molecular weight fraction. To study the glycosidic-linkage composition of the compounds formed upon an extreme roasting, namely those presenting higher molecular weight and those presenting lower molecular weight, the coffee galactomannans were inserted into an ashing furnace for a period of 3 h at 240 ºC, giving origin to fraction GM_240_F. The roasted material was then submitted to a dialysis, allowing obtain two fractions, corresponding to the high molecular weight material (HMWM) in the retentate, accounting for the major part of the galactomannan material, and the low molecular weight material (LMWM) that diffused to the dialysate.

Based on the methylation analyses (Table 4.2.1) of the fractions before (GM) and after the roasting process at 240 ºC (GM_240_F), it was observed that the roasted material was mainly composed by (1→4)-linked Man residues (94 mol% of all Man), with 4 and 2 mol% of terminally-linked and (1→4,6)-linked Man, showing a shorter mannan structure. In this fraction no galactose residues were obtained and a higher amount of (1→5)-linked Ara residues whose origin is unknown were also observed.

The dialysate fraction, corresponding to the low molecular weight material (GM_240_F_LMWM), when compared with GM, presented a higher amount of terminally-linked Man residues (49 mol%) and a lower amount of (1→4)-linked Man residues (42 mol%), characteristic of a low molecular weight material. These results allowed to conclude that even after an extreme roasting procedure, a major part of the polymeric material remains intact. When the roasting process occurs in a solid phase material, it seems that the degradation takes place mainly in the external part of material, protecting the material in the interior.

IV.2.4. Approaches to increase water solubility of roasted galactomannans

The coffee galactomannans isolated with alkali solutions are insoluble in water. Because the roasting process can promote depolymerisation and, consequently, solubilisation of galactomannans, the galactomannans were roasted at different temperatures and their solubility was tested. Upon roasting at 160 ºC during 1 h, it was possible to solubilise at room temperature only 0.7% of insoluble galactomannans, in this roasting sample. After 8 consecutive roasting and solubilisation procedures, only 3.3% of galactomannans have been solubilized. This low solubilisation in water confirms the high thermal stability of galactomannans at 160 ºC.

In order to improve the yield of solubilisation of galactomannans, three consecutive roasting at 200 ºC during 1h and solubilisation in water at room temperature (20 ºC) steps were performed (Scheme 2.2 in chapter II), allowing to obtain the fractions R1W20sn, R2W20sn, and R3W20sn. With this procedure 18.5% of galactomannans become soluble in water at room temperature. These soluble fractions, when compared to the initial insoluble galactomannan was shown to be composed by an higher amount of terminally-linked Man residues and lower amount of (1→4)-linked Man residues (Table 4.2.3), indicating the presence of lower molecular weight material. These three soluble fractions also showed higher relative amounts of (1→4,6)-linked Man residues, allowing to infer the occurrence of more branched compounds. In these fractions, although in a small extent, it was observed the presence of (1→3,6)-linked and (1→3)-linked Gal residues, which may represent co-solubilisation of arabinogalactans. When the remaining insoluble material was submitted to a solubilisation in water at 90 ºC it was possible to solubilise more 4.5%. Based on the glycosidic-linkage composition of this hot water solubilised galactomannan, a higher polymerization degree than those solubilized in water at 20 ºC, was observed. Also in this solubilized material it was observed the presence of a small amount of arabinogalactans.

Because a large extent of galactomannans still remained insoluble in water, a 4 M NaOH solubilisation was done, followed by neutralization and dialysis, allowing to obtain a fraction that remained soluble in water at room temperature, representing 51% of the initial insoluble material (R3NaOHsn). The glycosidic-linkage composition of R3NaOHsn is similar to the previous fractions, namely those obtained with water at 20 ºC, except for

the higher amount of (1→3,6)- and (1→3)-Gal, diagnostic of the presence of arabinogalactans. The insoluble residue that remained after the NaOH solubilisation, which accounted for 12.3% of initial galactomannan was neutralized and dialysed, allowing to obtain another water soluble galactomannan-rich faction (R3NaOHsnIR). The remaining insoluble material (R3NaOHIR) had also the glycosidic-linkage composition characteristic of galactomannans with a small portion of arabinogalactans, as well as a higher amount of (1→4)-Glc. The reason for the insolubility of the material should be related with the lower content in sugars.

Table 4.2.3 *Glycosidic-linkage composition (mol%) of galactomannan-rich solubilized fractions*

Linkage	GM	R1W20sn	R2W20sn	R3W20sn	R3W90sn	R3NaOHsn	R3NaOHsnIR	R3NaOHIR
T-Ara*f*	0.1	0.6	0.6	0.7	0.4	0.4	0.5	1.0
5-Ara*f*	0.5	0.2	0.2	0.1	0.0	0.2	0.2	0.3
T-Man*p*	1.7	9.9	12.1	17.6	9.3	7.1	7.1	13.0
4-Man*p*	90.1	78.1	73.1	65.7	82.2	76.2	69.7	56.4
4,6-Man*p*	1.5	4.5	7.0	9.0	5.2	7.4	6.9	4.5
T-Gal*p*	0.8	2.4	2.8	3.3	1.5	2.4	3.7	6.1
3-Gal*p*	1.1	1.8	1.8	1.4	0.5	2.5	4.5	7.2
3,6-Gal*p*	0.3	1.0	0.9	0.5	0.1	1.7	3.2	2.9
4-Glc*p*	4.0	1.6	1.6	1.6	0.7	2.2	4.3	8.6

IV.2.5. Coffee arabinogalactans

In order to evaluate the long term thermal resistance of coffee arabinogalactans, experiments were performed using isothermals at 160, 180, 200, 220, and 240 ºC during 3 h. Figure 4.2.1d presents the different isothermal weight loss curves of a coffee arabinogalactan along time, using the same procedure described for galactomannans (Fig 4.2.1c). It is possible to observe, as for galactomannans, two main mass loss stages, being the first mass loss, from 0 to 6 min, which can be attributed to the loss of water. This loss was approximately 5% for all isotherms except for 220 ºC and 240 ºC. The rate of loss of mass was 0.133 mg/min for 160 ºC, 180 ºC and 200 ºC assays. At 220 ºC the loss of water was not so well defined as the previous ones, possibly due to the simultaneous degradation of the polysaccharides at this stage. At 240 ºC it was observed a higher extent of mass loss

(23%) in the first 30 min of roasting. The mass loss that occurred from 6 to 210 min at all temperatures may be attributed to the polysaccharide decomposition. This loss was approximately 6% for experiments performed at 160 ºC and 180 ºC, 9% and 10% for the experiments performed at 200, 220 and 46% for the 240 ºC roasting. The rate of loss of mass ranged between 0.005 mg/min at 160 ºC and 0.033 mg/min at 240 ºC. These results show that the coffee arabinogalactans are thermally stable at temperatures lower or equal to 180 ºC during long term exposures up to 3 h, which is a temperature lower than the 200 ºC observed for coffee galactomannans. Taking into account that the coffee roasting process is usually performed at temperatures around 200 ºC, it is expectable that during the roasting of the coffee the arabinogalactans be degraded, contrarily to the galactomannans that keep their thermal stability. This is accordance with the lower values of activation energy of thermal decomposition determined for the arabinogalactans when compared with those of the galactomannans (Fig. 4.2.2c).

Glycosidic-linkage analysis of the arabinogalactan-rich material roasted at 160, 180 and 200 ºC (Table 4.2.4) showed that the (1→4)-Man, (1→4,6)-Man, and T-Man, which are diagnostic linkages for the presence of galactomannans, were similar, which are in accordance with the results previously described about the thermal stability of the galactomannans at these temperatures. On the contrary, the linkages diagnostic of the arabinogalactans do not show a consistent pattern, even after repetition of the methylation procedures. This may indicate a heterogeneous thermal lability of the arabinogalactans. Also, the samples showed a consistent increase of (1→4)-Glc with the increase of the roasting temperature. This may be explained by the degradation of the sugars and isomerization reactions that may take place (Nunes et al., 2006a). No sugars were observed in the samples AG_220 and AG_240, possibly due to the complete transformation of the arabinogalactan related sugars, or insolubilization of the arabinogalactans related material during the roasting process.

Table 4.2.4 *Glycosidic-linkage composition (mol%) of coffee by-product arabinogalactans rich fraction submitted to different thermal treatment at 160, 180 and 200 ºC.*

Linkage	AG	AG_160	AG_180	AG_200
T-Ara*f*	15.4	30.6	2.6	9.8
5-Ara*f*	2.7	3.0	2.5	0.0
T-Man*p*	1.6	2.0	1.7	0.0
4-Man*p*	23.5	22.1	21.3	17.6
4,6-Man*p*	0.3	0.0	0.0	0.0
T-Gal*p*	11.0	14.8	9.2	13.3
3-Gal*p*	29.6	21.2	20.9	15.2
6-Gal*p*	3.4	1.5	6.0	0.0
3,6-Gal*p*	11.2	2.0	12.5	0.0
4-Glc*p*	1.3	6.7	23.2	53.9

IV.2.6. Concluding remarks

The thermogravimetric study performed showed that the roasting at 200ºC up to 3 h promoted structural changes of the coffee residue galactomannans with no apparent weight loss. Compared with the coffee residue arabinogalactans, the galactomannans were more resistant to weight loss at this temperature. However, at 180ºC the arabinogalactans were also thermally stable up to 3 h. This different thermal stability was in accordance with their activation of energy for thermal degradation, which were 138 kJ/mol for the coffee galactomannan and 94 kJ/mol for the coffee arabinogalactan. This higher heat lability of arabinogalactans should the responsible for the lower activation energy of thermal decomposition of coffee by-product, while the galactomannans and cellulose should confer higher resistance. Although not leading to significant mass loss, the roast of coffee galactomannans promoted transglycosylation, as inferred by the formation of new linkages such as (1→2)- and (1→6)- linked mannose residues, formation of anhydrohexose residues at the reducing end of the polysaccharides, depolymerisation, and isomerisation reactions by formation of (1→4)-linked glucose residues. These modifications of coffee galactomannans allowed their solubility in water upon alkali extraction and neutralisation.

CHAPTER V

CONCLUSIONS

The detailed study of the structural features of four galactomannans (coffee residue, coffee infusion, LBG and *Aloe vera* galactomannans), three of them presenting immunostimulatory activity (coffee residue, coffee infusion and *Aloe vera* galactomannans), showed similar patterns, namely the presence of single arabinose residues and acetyl groups in all of them, even in LBG galactomannan, where these components have never been reported. However, several differences were also determined, namely, the degree of acetylation, that was much higher in *Aloe vera* than in coffee infusion galactomannans. Similar degree of branching and molecular weight were found for the galactomannans presenting immunostimulatory activity, whereas the galactomannan with no immunostimulatory activity presented a higher degree of branching (Total Man*p*/4,6-Man*p* ranging = 4) and degree of polymerization (Total Man*p*/T-Man*p* = 68). Lower branching (Total Man*p*/4,6-Man*p* ranging from 13 to 28), shorter chains (Total Man*p*/T-Man*p* ranging from 20 to 26), and some acetylation (AcOH/sugar residues ranging from 2.08 to 0.08) seem to promote the immunostimulatory activity of these polysaccharides.

The reutilization of coffee by-product is promising by its application as a source of galactomannans with immunostimulatory activity. Roasting of coffee residue at 160 ºC complemented with hot alkali extractions increased the yields of extraction of galactomannans, without their apparent degradation by loss of matter. An extreme roasting of galactomannans, at 220ºC, promoted the loss of a large extent of mannans, however, their structural features and even the small details such as acetylation and galactose and arabinose side chains are kept. These results allow to infer that coffee residue galactomannans have a high resistance to temperature.

The galactomannans of coffee by-product, when subjected to an isothermal treatment at T ≤ 200 ºC during exposures up to 3 h did not loss its mass whereas the arabinogalactans achieved the thermal stability only for T ≤ 180 ºC. The coffee residue galactomannans are more thermal stable and less heat labile than coffee residue arabinogalactans. Coffee residue galactomannans roasted at 200 ºC did not loss mass but some structural features changed. These roasting structural changes involved the formation of new glycosidic linkages, namelly the 2- and 6-linked mannose residues, transglycosylation of polysaccharides forming new structures, and depolymerisation,

allowing to obtain galactomannan-derived oligosaccharides. Also, roasting of galactomanans allows the formation of anhydrohexose residues at the reducing end, and isomerisation of mannose residues to glucose. The roasting of insoluble galactomannans at 200 ºC promoted their solubility in water upon alkali extraction and neutralisation.

To extract the galactomannans from coffee residue a roasting treatment of the coffee residue accompanied by hot alkali treatments can provide high yields, up to 55%. These galactomannans, which are majorly insoluble, can be rendered soluble in cold water in an extent of 74% if submitted to a roasting process at 200 ºC. It is promising that these extracted and solubilized galactomannans can be used as a source of galactomannans with immunostimulatory activities. Based on the previous structure-function studies, as a future work, the structure of the polysaccharides obtained should be optimized according to the parameters that were shown to influence positively the immunostimulatory activity, namely, the degrees of branching and polymerization, and the degree of acetylation. These should be complemented with new trials of evaluation of the immunostimulatory activity of coffee residue galactomannans, first *in vitro* and, when succeeded, by *in vivo* animal and human trials.

CHAPTER VI

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